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Amylin sensitivity and secretion following diet-induced obesity in rats

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Contents

1 Summary

The aim of this study was to investigate the influence of diet-induced obesity, and the resulting changes in hormone metabolism, on amylin sensitivity and secretion.

Our investigation resulted in the following findings and conclusions:

First study

Findings: After eleven weeks on high fat-feeding, obese rats showed an attentuated anorectic response to exogenous amylin (5µg/kg). Further, high fat-feeding increased amylin and leptin baseline levels as well as total, visceral and subcutaneous body fat.

Conclusion: Long-term high fat-feeding decreases sensitivity to peripheral amylin in obese rats.

Second study

Findings: Following chronic elevation of circulating amylin levels in non-obese rats, changes in amylin sensitivity to the acute anorectic effect of amylin were not observed. Exogenous amylin administration decreased food intake dose-dependently, regardless of circulating amylin levels, at least within the first hour after administration.

Conclusion: Hyperamylinemia, without the influence of obesity, does not seem to reduce amylin's anorectic effect.

Third study

Findings: After eight weeks on a high fat diet, diet-induced obese and diet resistant rats showed changes in meal induced amylin and insulin secretion pattern. In addition, baseline amylin, leptin and insulin levels increased with an increase in body weight.

Conclusion: The differences in meal-induced amylin secretion seem to be influenced more by the composition of the diet than by the higher body weight of obese rats. On the other hand, mealtime insulin secretion is likely to be influenced more by actual body weight or fat mass.

2 Introduction

2.1 The Obesity Epidemic

Obesity, which has reached epidemic proportions globally, is typically the result of an imbalance between energy intake and energy expenditure. Once associated with high-income countries, obesity is now also prevalent in low- and middle-income societies. The primary reasons for the global increase in overweight and obesity are the shift toward increased intake of an energy-dense diet that is high in fat and sugar, and a trend toward decreased physical activity. According to the World Health Organization (WHO), approximately 1.6 billion adults were overweight and at least 400 million adults were obese in 2005. Additionally, in 2005 there were at least 20 million children under the age of five who were overweight. Worldwide there are more people who die from complications of obesity than from being underweight or malnourished. This demonstrates the significance of this disease and its consequences (Flegal et al., 2007).

Several health problems associated with obesity are frequently classified as symptoms of metabolic syndrome, including the development of insulin resistance, type 2 diabetes, fatty liver disease and cardiovascular disease. According to the WHO, more than 220 million people worldwide have diabetes, approximately 90% of which have type 2 diabetes; type 2 diabetes is largely the result of excess body fat and physical inactivity (Katz et al., 2000). Some other consequences of obesity or type 2 diabetes include an increased risk of nephropathy, retinopathy, osteoarthritis, respiratory disease, sleep apnea and several types of cancer, as well as an increased general risk of mortality (Pi-Sunyer, 2002; Kesty et al., 2008).

2.2 Current Therapeutic Approaches

Prevention and early treatment of overweight and obesity can often be achieved by maintaining or restoring the balance between calories consumed and calories expended. In addition to a change in diet, physical activity may also be beneficial for weight control. In most cases of overweight and obesity, however, diet and increased exercise do not produce sufficient weight loss. Bariatric surgical interventions, such as gastric bypass or gastric banding, are the most successful weight loss treatments (Dixon et al., 2005; le Roux et al., 2007). However, such treatments are invasive and expensive, and are typically only

recommended to patients suffering from at least Class II obesity, with a BMI of 35 or greater (Rubino et al., 2004; Moo and Rubino, 2008). For overweight or obese patients with a BMI less than 35, pharmacological treatment is an alternate approach to weight loss management. While significant advances have been made in the development of potential drugs that can manage body weight as well as diabetes, subsequent progress is limited by not fully understanding the central and peripheral mechanisms that control energy homeostasis. Furthermore, we must also consider the potential consequences that the physical state of obesity may intrinsically have on the success of such therapies.

It is known that numerous metabolic, neuronal and endocrine signals are involved in controlling energy homeostasis and therefore also influence the development of obesity.
Long-term adiposity (tonic) signals that represent the energy store of the body, such as leptin and insulin, and short term peripheral satiation (phasic) signals that are produced in response to the current nutritive state, such as amylin, CCK and GLP-1 are a few examples discussed below (Kesty et al., 2008).

2.3 Controls of Energy Homeostasis

Body mass and composition are a reflection of three processes of energy homeostasis: energy intake, energy storage and energy expenditure. Energy homeostasis is achieved when energy intake equals energy output, and obesity, or excess energy storage, results when energy consumption exceeds energy expenditure. Metabolic, neuronal and endocrine signals are involved in controlling energy homeostasis and these also play a role in the development of obesity.

Most humans eat the same approximate numbers of meals, at a similar time of the day, on a daily basis. A variety of factors control meal onset and meal size (Woods, 2005). Social influences, emotions, time of day, even the convenience or the cost of food can lead to variability in daily energy intake within and among individuals (Edholm, 1977). In addition to these non-biological factors, a network of biological processes tightly controls energy homeostasis. The hormones and peptides, from both the periphery and the central nervous system, that converge in the brain to control meal-to-meal food intake are typically classified as hunger, satiation, or satiety signals. Another class of hormones, called adiposity signals, are

secreted in proportion to body fat and are thought to control food intake and body weight in the long-term in order to maintain stable body weight.

2.3.1 Peripheral signals

2.3.1.1 Hunger signals

Identified in 1999, ghrelin is the only known peripheral peptide that stimulates appetite (Cui et al., 2008; Perboni and Inui, 2009). Ghrelin is a gastrointestinal (GI) hormone secreted from the enteroendocrine cells of the stomach, the small intestine and the colon into the portal vein (Kojima et al., 1999). It was recently suggested that ghrelin may act as an inverse adiposity signal, because plasma concentrations are low in obese individuals, and a loss of body weight in overweight humans is accompanied by an increase in circulating ghrelin concentration (Tschop et al., 2001; Hansen et al., 2002). Primarily linked to food intake, ghrelin levels rise and fall throughout the day. High ghrelin levels are observed following fasting or in anticipation of a meal, and levels decline after energy intake (Drazen et al., 2006; Cui et al., 2008). However, the relevance of ghrelin as a physiological signal to trigger meal onset is unknown.

2.3.1.2 Satiation and Satiety signals

Satiation signals are released following meal initiation to limit meal size. During food consumption, most satiation signals are secreted and then act by either stimulating vagal afferents that project to the hindbrain or by stimulating the hindbrain directly. From here, the signals are relayed to other brain areas, where they are integrated with adiposity signals, as well as with information encoding hedonic and social factors, to eventually terminate eating (Woods, 2009).

Cholecystokinin (CCK), which is mainly produced in the duodenum and jejunum, and is rapidly released into the circulation in response to local nutrients, is considered the prototypical satiation signal (Gibbs et al., 1973; Grider, 1994). CCK acts on the vagal nerve, which projects to the nucleus of the solitary tract (NTS) in the hindbrain, and possibly also directly on the CNS by crossing the blood-brain barrier (MacLean, 1985; Edwards et al., 1986). Administration of CCK to humans and animals inhibits food intake by reducing meal

size and duration (Lieverse et al., 1995; Gibbs et al., 1997). However, because of CCK's short half-life (approximately 1-2 min), and because continuous infusion of CCK becomes ineffective in decreasing meal size within 24 hours (Crawley and Beinfeld, 1983), CCK alone does not appear to be therapeutically relevant for the treatment of human obesity (Wynne et al., 2005), unless, perhaps, when the number of meals is strictly controled.

Another well studied satiation signal is glucagon-like peptide-1 (GLP-1), which is released during the meal from the L-cells of the GI tract and peaks post-prandially in proportion to the amount of energy ingested. GLP-1 is also produced in some neurons in the NTS (Chaudhri et al., 2008). GLP-1 reduces food intake when administered either peripherally or centrally (Baggio et al., 2004; Abbott et al., 2005). It has been demonstrated that i.p. infusion of GLP-1 selectively reduced the duration and the size of the ongoing meal, without affecting the intermeal interval and the size of subsequent meals (Ruttimann et al., 2009). With a short half-life of approximately 2 minutes, GLP-1 produces only a transient reduction in food intake when administered peripherally (Baggio and Drucker, 2007). However, GLP-1 receptor agonists designed with half-lives up to 8 days show promising potential for treatment of obesity or type 2 diabetes (Baggio et al., 2008).

Upon termination of a meal, a state of non-eating, or satiety, begins and continues until the next meal is initiated. This state is regulated by satiety signals (Williams et al., 2009).

In addition to CCK and GLP-1, gastrin releasing peptide (Stein and Woods, 1982), neuromedin B (Ladenheim et al., 1996), enterostatin (Shargill et al., 1991), somatostatin (Lotter et al., 1981), apolipoprotein A-IV (Fujimoto et al., 1993) and peptide YY (Batterham et al., 2002) are also peptides secreted from the GI tract that have been shown to reduce meal size and which may perhaps also induce satiety when administered systemically. However, the latter effect needs to be studied in more detail.

2.3.1.3 Adiposity signals

Adiposity signals are hormones that are secreted in proportion to body fat; increased circulating levels of adiposity signals are indicative of a larger amount of stored fat. The two best investigated and most prominent adiposity signals are leptin and insulin. In normal-weight animals, a change in circulating adiposity signals signifies a change in body fat stores, and typically results in a behavioral response. For instance, an increase in basal leptin or insulin would indicate a gain in body weight, and the animal would respond by consuming

less food to restore a lower body weight. When administered to an overweight or obese animal or human, however, these individuals are relatively insensitive to the food intake- and weight-reducing effects of adiposity signals (Woods, 2009). Thus, while obesity is associated with hyperinsulinemia and hyperleptinemia, it also generates resistance to these signals, which challenges their theurapeutic usefulness.

Adipocyte-derived adiposity signals

Leptin is a cytokine that is expressed and secreted primarily by adipocytes. Leptin levels increase with weight gain, and fasting levels are directly proportional to adipose mass (Maffei et al., 1995). Because fat cell size is usually enlarged 2 to 4 times in obese individuals, obesity can result in a seven-fold increase in leptin secretion compared to that seen in lean individuals (Fried et al., 2000).

The plasma protein adiponectin is secreted exclusively from adipose tissue (Hu et al., 1996). Adiponectin may be considered an inverse adiposity signal because unlike leptin, there is typically a strong negative correlation between the plasma adiponectin concentration and fat mass (Hu et al., 1996). Hence, while obesity lowers adiponectin levels, weight reduction increases circulating adiponectin. Adiponectin has been shown to improve the whole-body insulin sensitivity in models of genetic and diet-induced obesity (Combs et al., 2001). It is thought to stimulate fatty acid oxidation and glucose uptake in skeletal muscle and adipose tissue, thereby attenuating body-weight gain without affecting food intake (Berg et al., 2001; Yamauchi et al., 2002; Wu et al., 2003).

Pancreatic ß-cell-derived adiposity signals

Insulin demonstrably fulfils the criteria of a long-term adiposity signal, namely that observed plasma levels are positively correlated with body fat (Bagdade et al., 1967). While leptin is disproportionately secreted from subcutaneous fat, the insulin secretion more closely reflects the amount of visceral fat mass, thereby acting as a negative feedback signal (Considine et al., 1996). As with leptin, weight gain reduces insulin sensitivity, thus elevated insulin secretion in both the basal state and in response to meals is required if normal glucose homeostasis is to be maintained (Clegg et al., 2005). It has been shown in rats that insulin is secreted in a high-frequency pulsatile manner (Matveyenko et al., 2008), and that during fasting, the liver is

exposed to insulin oscillations of ~400-600 pmol/l (Matveyenko et al., 2008). Hepatic insulin clearance is directly related to the amplitude of insulin pulses in the portal vein. Because approximately 80% of insulin is cleared in the first pass through the liver, systemic insulin concentrations may underestimate changes in insulin secretion, especially pulsatile insulin secretion (Matveyenko et al., 2008).

2.3.1.4 Amylin

The second peptide produced and secreted by the pancreatic ß-cells is amylin, which is the focus of this dissertation. Amylin, also known as islet amyloid polypeptide (IAPP), is co-secreted with insulin in response to nutrient consumption (Ludvik et al., 1997). Amylin shares the typical characteristics of satiating hormones, like CCK, and plays a role in the control of meal size. Thus, in response to nutrient ingestion, circulating amylin concentrations rise rapidly, i.e. within minutes after meal onset, and peak within 60 min and then remain elevated for up to 4 hours after a typical meal (Koda et al., 1992). When administered exogenously (peripheral or central), amylin dose-dependently decreases food intake, causing a decrease in meal size though having no effect on the intermeal interval (Lutz et al., 1995; Rushing et al., 2001). Furthermore, the decrease in meal size is not a result of an aversive or toxic effect of amylin (Lutz et al., 1995; Mack et al., 2007).

In addition to functioning as a satiation signal, amylin also meets the main criteria to be considered an adiposity signal. Similar to leptin and insulin, the basal plasma level of amylin is directly proportional to body adiposity (Pieber et al., 1994), with elevated fasting amylin concentrations in obese individuals that increase further with weight gain (Kesty et al., 2008). It has been shown that chronic subcutaneous administration of low doses of amylin to ad libitum fed rats reduces food intake and body weight gain (Arnelo et al., 1996; Arnelo et al., 1998), and also causes a reduction in fat depot size (Isaksson et al., 2005). Additionally, a chronic infusion of amylin antagonists increases body weight and body fat mass (Rushing et al., 2001). These observations are consistent with the concept that amylin acts as an adiposity signal to control energy homeostasis.

2.3.2 Central processing of peripheral signals that are involved in the control of eating

The brain receives and integrates multiple forms of information from numerous organs that are involved in energy homeostasis. Information regarding energy stores and fluxes in critical organs, the ingestion of food, and the basal and current energy need by various tissues is processed by the brain to maintain homeostasis. The primary brain nuclei regulating energy balance are located in the hindbrain and the hypothalamus.

2.3.2.1 The hindbrain

The hindbrain, specifically the area postrema (AP) and the nucleus of the solitary tract (NTS), plays an important role in the control of food intake and body weight. It is a major site of abdominal sensory input to the central nervous system, to which vagal and splanchnic afferents from the abdominal viscera project directly or indirectly (Hyde and Miselis, 1983). Furthermore, the AP is a circumventricular organ, and hence lacks a functional blood brain barrier; this allows signals in the blood to directly access the receptors in the AP (Lutz et al., 1998). Receptors for several satiation and adiposity signals are localized in the AP and NTS, including amylin, CCK, GLP-1, insulin, and leptin (Woods and D'Alessio, 2008). Neuronal populations expressing these receptors then convey information to various other brain regions. The AP has efferent projections to the NTS, the dorsal motor nuclei of vagus and the lateral parabrachial nuclei, whereas the NTS has efferent projections to the hypothalamus and limbic structures (Ricardo and Koh, 1978). Hypothalamic nuclei also send reciprocal projections back to the AP and the NTS (van der Kooy et al., 1984). Due to the presence of such receptors and neuronal connections, the AP/NTS has been proposed to act as a central receiver and processor of metabolic and humoral signals (Hyde and Miselis, 1983). This notion is supported by the fact that lesions of the AP/NTS induce a syndrome of hyperphagia in rats, which consequently gain more weight (Hyde and Miselis, 1983). The increase in body weight suggests a loss of visceral sensory input to various receptors both in and downstream of the AP/NTS, thus impairing homeostatic control on several levels.

2.3.2.2 The hypothalamus

While the hindbrain, containing motor output circuits and the neuronal processing for the control of meal size, plays a critical role in food intake, regulation of motivated ingestive

behavior requires contribution of the hypothalamus, which is situated in the medial basal forebrain (Swanson, 2000). Over half a century ago, seminal lesion studies identified the ventromedial hypothalamic nucleus (VMN) as the "satiety center," and the lateral hypothalamic area (LHA) as the "hunger center" (Hetherington and Ranson, 1940; Anand and Brobeck, 1951). Even though this view is far too simplified, these nuclei remain important in energy homeostasis control. However, in recent years, and notably following the discovery of leptin in 1994 (Zhang et al., 1994; Halaas et al., 1995), considerable focus has been placed on the arcuate nucleus (ARC) as the central hypothalamic control of food intake.

The ARC, which is located in the ventral hypothalamus, along with the median eminentia (Rethelyi, 1984), has a relatively leaky blood-brain barrier and expresses several important receptors that bind peptides involved in the control of energy homeostasis. Because of this, the ARC is ideally situated to gauge the circulating levels of adiposity signals. Upon binding to the ARC, leptin and insulin interact with two neuronal populations that synthesize either proopiomelanocortin (POMC) or neuropeptide Y (NPY) and Agouti-related peptide (AgRP); both populations are involved in energy homeostasis. POMC is cleaved to produce the neurotransmitter α-melanocyte stimulating hormone (α-MSH), which reduces food intake by activating melanocortin 3 (MC3R) and melanocortin 4 (MC4R) receptors found on neurons in other hypothalamic regions and elsewhere in the brain (Schwartz et al., 2000; Cone, 2005). NPY, on the other hand, acts at Y receptors to stimulate food intake (Stanley et al., 2005). AgRP is an endogenous MC3R and MC4R antagonist and therefore also acts as an orexigenic agent, countering the activity of POMC neurons (Cone, 2005). These neuronal populations project namely to the paraventricular nucleus (PVN) and the LHA, to further modulate food intake and energy expenditure. Stimulation of the PVN typically leads to a catabolic action that reduces food intake and increases energy expenditure, whereas stimulation of the LHA results in an anabolic process that increases food intake and body weight gain (Woods and D'Alessio, 2008).

The long-form of the leptin receptor (ObR-L) is present in high concentrations in the ARC (Schwartz et al., 1996; Elmquist et al., 1998), and it has been shown that leptin reduces food intake and controls energy expenditure by activating POMC neurons and inhibiting NPY neurons (Elias et al., 1999). Insulin receptors are also located in the ARC and, like leptin, insulin stimulates the hypothalamic melanocortin system to carry out its catabolic action (Benoit et al., 2002). Finally, the orexigenic effect of ghrelin is mediated by the activation of

ARC neurons that co-express NPY and AgRP, and the indirect inhibition of POMC-containing neurons (Riediger et al., 2003). These, and other data suggest that ghrelin acts in a functionally antagonistic manner to leptin and insulin in the ARC (Traebert et al., 2002).

2.3.2.3 Central processing of amylin

Specific amylin binding sites have been identified in various discrete brain nucluei in the telencephalon, hypothalamus and hindbrain (Sexton et al., 1994). Functional amylin receptors are composed of a calcitonin receptor (CT-R) core and receptor-activity modifying proteins (Christopoulos et al., 1999), normally RAMP1 or RAMP3. RAMPs regulate transport of core receptors to the cell surface, and are involved in ligand specificity of the receptor. All necessary components for the amylin receptor are located in the AP, which is thought to be the primary central target of circulating amylin (Lutz et al., 1998; Ueda et al., 2001).

Lesion and immunohistochemical studies further confirm the importance of the AP in mediating amylin's actions. Immunohistochemical detection of c-Fos expression is a commonly-used technique for mapping neuronal activation in the brain (Curran and Morgan, 1995). Both exogenously applied and feeding induced endogenous amylin induces c-Fos in the AP/NTS region, as well as in other brain areas such as the lateral parabrachial nucleus (LPBN) and the central nucleus of the amygdala (Riediger et al., 2004). Lesions of the AP/NTS, but not blockade of neural afferents projecting to the brain, abolish the anorectic effect of amylin (Lutz et al., 1998). Lesions of the AP have also been shown to attenuate the c-Fos response in the NTS, LPBN, and CeA, demonstrating the key role that the AP plays in mediating amylin's action (Rowland and Richmond, 1999; Riediger et al., 2004).

2.4 Obesity and Modified Homeostatic Control

2.4.1 Rodent models of obesity

To study how the obese state alters homeostatic regulation, numerous rodent models of obesity have been developed over the past half-century. Obesity in rats can result from several factors, including spontaneous mutations, targeted, genetic modification, and environment (e.g. access to a diet high in fat). Such animal models are very important for studies probing the environmental influences on obesity (e.g. epigenetics), the responses to

diets containing various levels of fat, carbohydrates, protein, or calories, and the identification and development of pharmaceuticals for obesity treatment. Today there are several well-established animals models available that provided an enormous amount of information about obesity. Models for physiological and genetic basis of obesity included e.g. the leptin-deficient *ob/ob* mouse, the Zucker *fa/fa* rat and the Otsuka Long Evans Tokushima Fatty (OLETF) rat, which is CCK-1 receptor-deficient. The diet-induced obesity (DIO) rat model (Levin et al., 1997), which was used in the studies described below, is frequently used in research because it mimics the more common form of obesity in humans that is not caused by a specific mono-genetic mutation.

Zucker fa/fa rats

The Zucker-fatty rat was observed following a spontaneous mutation; fa/fa homozygous animals suffer from genetic obesity, and progressively accumulate fat mass, which is predominately subcutaneous, over their lifespan (Argiles, 1989; Moran, 2008). The *fa* gene mutation prevents expression of the long isoform of the leptin receptor (Phillips et al, 1996). Hyperphagia is one of the main characteristics of the obese Zucker-fatty rat (Bray and York, 1972), however, the fact that pair-feeding obese rats with lean rats reduces, but does not prevent, the development of obesity, suggests that hyperphagia is not the only factor causing increased fat storage but that decreased energy expenditure may also be involved (Pullar and Webster, 1974). The obese Zucker rats are characterized as having hyperinsulinemia, which results from both hypertrophy and hyperplasia of the pancreatic ß-cells (Zucker and Antoniades, 1972), and from reduced hepatic clearance of the hormone (Argiles, 1989). Zucker-fatty rats are also glucagon resistant. Despite having normal pancreatic glucagon levels, basal plasma glucagon and plasma glucagon concentrations in response to fasting are decreased (Eaton et al., 1976; Argiles, 1989). Obese Zucker rats are one of the best known and most widely used animal model for genetic obestity. Furthermore the Zucker diabetic fatty (ZDF) male rat is a rodent model of diabetes type 2 (Tokuyama et al., 1995).

OLETF rats

Obesity is also observed in Otsuka Long Evans Tokushima Fatty (OLETF) rats, which lack a functional CCK-1 receptor. Insensitivity to CCK leads to hyperphagia that is a consequence of a significant increase in meal size (Bi and Moran, 2002; Moran, 2008). OLETF rats

consume about 30% more than control Long Evans Tokushima Otsuka (LETO) rats, and fat deposition is predominately intra-abdominal or visceral, which is different from some other rat models (Kawano et al., 1994; Bi et al., 2001).
OLETF rats were also used as a model of type 2 diabetes and are characterized by late onset of hyperglycemia, polyuria, polydipsia and mild obesity (Kawano et al., 1994; Moran, 2008).

Diet-induced obesity (DIO) and resistance in Sprague-Dawley rats

A third rodent model of obesity, which was utilized in experiments conducted for this dissertation, is the DIO rat. As observed and described extensively by Levin and colleagues, when outbred Sprague-Dawley (SD) rats are fed a diet relatively high in fat, sucrose and energy density, approximately one-half will develop DIO, while the other half remain diet resistant (DR; (Levin et al., 1997). DR rats gain body weight and fat mass at a rate similar to that observed in chow-fed control rats (Levin and Keesey, 1998). Despite the fact that high-fat diets are generally known to stimulate insulin resistance in rats, only the DIO animals developed glucose intolerance and relative insulin resistance on high-energy (HE) diets. In addition to investigating the effects of diet on outbred SD rats, DIO and DR rats have also been selectively bred, which allows for the study of multi-genetic and experimental factors that might predispose an individual to obesity, prior to consumption of a high-fat diet (Ricci and Levin, 2003).

Rodent DIO is an appealing model because it shares several characteristics with common forms of human obesity. Namely, and unlike the obese Zucker or OLFTF rats, it is known that the bimodal pattern of body weight development in SD rats fed a HE diet is not due to a modification in a single gene (Levin et al., 1997). Like most cases of human obesity, the DIO model results from a combination of both inherited and environmental factors. Induction of complete DIO is dependent on access to a HE diet and is not observed if outbred animals are maintained on standard chow. Prior to HE diet exposure, DIO- and DR-prone rats show few phenotypic differences in peripheral metabolism. While total energy intake, body weight, motor activity, thermogenesis, and insulin sensitivity are similar between the two groups (Levin et al., 1997), a difference in patterns of food intake has been observed. Pre-obese, selectively-bred DIO rats fed a standard chow diet consume smaller, but more frequent meals (Cottone et al., 2007), reminiscent of human snacking behavior, which may predict adult obesity (Erlanson-Albertsson, 2005). This suggests that even prior to HE excess, the DIO rats

are genetically predisposed to become obese. Furthermore, DIO prone rats also show a generalized increase in sympathetic activity when chow-fed, and develop glucose intolerance and hyperinsulinemia after only two weeks on high energy diet (Levin et al., 1997). Selectively bred DIO rats also demonstrate increased feed efficiency when fed HE diet compared to DR rats (Levin and Dunn-Meynell, 2000). As a result of this heightened metabolic efficiency, excess energy retention is deposited with high efficiency in adipose tissue (Levin et al., 1997).

DIO and DR rats also respond differently to various metabolic challenges. When DIO rats are weight reduced by caloric restriction after consuming a variety of palatable and/or high energy diets, there is a relatively rapid return to the obese baseline once they are allowed free access to almost any diet. DIO restricted rats (50% of baseline intake) reduce their fat pad weights, plasma leptin, and insulin levels during caloric restriction. Chronic caloric restriction results primarly in loss of carcass fat in DIO rats, whereas DR rats primarly lose lean body mass (Levin and Dunn-Meynell, 2000). It has also been shown that outbred and selectively-bred DIO rats have similar reduction in central anorectic response to insulin compared to DR rats. Even though DR do not become obese or hyperinsulinemic on high-energy diet, they develop a considerably diminished anorectic response to central insulin administration after four weeks on this diet (Clegg et al., 2005).

2.5. Treatment of Obesity and Amylin

The use of rodent models of obesity has played a pivotal role in the exploration and development of pharmacological treatments of obesity and related diseases. Specifically, utilization of the DIO rodent model has revealed the therapeutic potential of amylin as a anti-obesity agent. Unlike CCK, which loses efficacy over time under ad libitum feeding conditions, chronic amylin treatment in DIO rats reduced food intake and body weight for up to eleven weeks (Mack et al., 2007). The weight-reducing effect is dose-dependent, and predominantly targets loss of fat mass, while preserving lean mass. While decreased food intake is believed to be the primary contributor to weight loss, pair-fed control rats do not lose as much weight as amylin-treated rats, suggesting that amylin may act in other ways to potentiate weight reduction (Roth et al., 2006) possibely by an effect on energy expenditure (Wielinga et al., 2007; Wielinga et al., 2010). Similar results have also been observed in obese humans. Obese individuals treated with the amylin analogue, pramlintide, demonstrated

significant weight loss, which was further enhanced by supplemental lifestyle changes (Smith et al., 2007; Smith et al., 2008).

While these data demonstrate the potential importance of amylin as a treatment for obesity, more recent evidence shows that amylin therapy can become even more powerful when partnered with a second or third hormone-based treatment. For instance, DIO rats that are treated with leptin in addition to amylin, show a synergistic reduction in body weight and fat mass that is significantly greater than the additive effect of the two treatments (Roth et al., 2008; Trevaskis et al., 2008). Researchers have suggested that this effect is due to an amylin-mediated restoration of leptin-responsiveness in DIO rodents. This notion is supported by the fact that pretreatment with amylin restores leptin-induced intracellular signaling (as determined by pSTAT3 immunoreactivity), which is lost in DIO rats (Roth et al., 2008). Amylin and leptin treatment also enhances leptin binding in hypothalamic regions, such as the VMN and ARC. Furthermore, amylin knockout mice show attenuated hypothalamic leptin-induced pSTAT3 staining, again suggesting the interdependence of these two hormones (Turek et al., 2010). This synergy has also been observed in human trials. In a clinical trial performed by Amylin Pharmaceuticals, coadministration of amylin and leptin for 24 weeks produced weight loss of 12.7%, which was significantly greater than either treatment alone (Roth et al., 2008; Ravussin et al., 2009).

2.6. Hypotheses and Aims of the Dissertation

The approval of the amylin analog pramlintide (Symlin®) for clinical use in diabetes treatment in the United States is an important step forward. Interestingly, compared to diabetics who are only treated with insulin, pramlintide and insulin co-therapy leads to body weight reduction. Nonetheless, the mechanisms underlying the role of amylin as both a satiation and adiposity signal, specifically in obese subjects, still require intensive study. In fact, it has been shown that in some rodent models of obesity (e.g. *ob/ob* mice, *db/db* mice, *fa/fa* rats, MC4Rko mice and DIO rats), higher doses of amylin are required to reduce eating (Eiden et al., 2002; Roth et al., 2006). Additionally, clinical tests report that higher doses of pramlintide are necessary to promote weight loss in type 2 versus type 1 diabetics, suggesting that amylin deficiency, as found in type 1 diabetics, may perhaps increase the efficacy of amylin treatment (Buse et al., 2002). Based on these data, the overarching goal of this dissertation is to the test the hypothesis that obesity reduces the sensitivity to the anorectic effects of amylin. A subset of this goal was to determine if factors associated with obesity,

such as consumption of a high-fat diet or hyperamylinemia, also lead to a change in amylin sensitivity, independently of obesity. Additionally, we investigated how obesity affects the meal-induced secretion of amylin. Based on these aims, we performed three studies directed at answering the following questions:

1.) Does body composition influence the anorectic effect of amylin?

In the first experiment, we tested whether the effect of amylin to reduce eating is affected by body composition in lean and obese rats. Modifications to rats' body composition were induced either by food restriction or by maintenance on a high fat diet. Rats provided long-term access to a high fat diet showed hyperamylinemia, hyperleptinemia and hyperinsulinemia. Because it is known that increases in circulating leptin and insulin levels contribute to leptin and insulin resistance, we hypothesized that diet-induced obesity, or the resulting hyperamylinema, decreases the sensitivity to the anorectic effects of exogenous amylin. In contrast, we hypothesized that food restricted rats with low circulating amylin levels would demonstrate heightened amylin sensitivity.

2.) Does chronic peripheral elevation of amylin levels induce a change in amylin sensitivity?

In the second experiment, we investigated if hyperamylinemia, which typically results from obesity, has an influence on the anorectic response of amylin independent of obesity. Previous studies had suggested that high circulating amylin levels reduce the sensitivity to amylin's ability to slow gastric emptying (Pieber et al., 1994; Young, 2005). Thus, we hypothesized that in animals, in which circulating baseline amylin was increased through chronic amylin administration, the sensitivity to acute exogenous amylin administration may be decreased. Furthermore, we hypothesized that the reduction in amylin sensitivity is proportional to the amylin concentration of chronically infused rats.

3.) Is the amylin secretion pattern, as measured from the portal vein, after a test meal, altered in diet-induced obese rats?

Based on data demonstrating that obese individuals have higher baseline amylin levels (Pieber et al., 1994), our final experiment focused on the pattern of meal-induced amylin release from the pancreas in obese and lean rats. Using the DIO/DR bimodal pattern of inheritance

observed in Sprague-Dawley rats fed a HE diet (Levin et al., 1997), we investigated whether diet composition or resulting DIO phenotype affects the release of amylin during a test meal. As an internal control, we also measured meal-induced insulin release, which allowed us to calculate the amylin:insulin concentration ratio, which is typically between 0.01 and 0.03 (Alam et al., 1992; Blackard et al., 1994). All animals were fitted with hepatic portal vein catheters (HPV), which allow for the collection of blood samples directly from the point where the pancreatic branch joins with the portal vein. We hypothesized that meal-induced release of amylin differs between DR, DIO or chow-fed rats.

3 Animals, Material And Methods

3.1 Animals

For all experiments, Sprague-Dawley rats (initial body weight 240-300g; Harlan NM Horst, the Netherlands) were used. A part of the animals served as a model for diet-induced obesity (DIO). The animals were individually-housed in hanging, stainless steel wire-mesh cages and were maintained in a temperature-controlled environment (21± 2°C), on a 12/12h light-dark cycle. Water and food were accessible ad libitum, unless otherwise indicated.

All rats were habituated to the housing conditions for at least one week prior to the start of an experiment. During habituation, rats were handled daily, and they were also allowed 30 min access to a common, enriched environment to play. All experiments were approved by the Veterinary Office of the Canton of Zürich, Switzerland.

3.2 Experimental Diets

Standard pelleted chow:

During the acclimatization phase of each experiment and during the entire duration of the second experiment, rats were allowed ad libitum access to standard pelleted chow (Diet 3430, Provimi Kliba AG, Kaiseraugst, Switzerland; metabolisable energy content: 13.2 kJ/g, protein(w/w): 21%, carbohydrates: 39.8%, fat: 5%). This standard chow was also fed to control animals in experiments one and three.

High-fat pelleted diet:

In the first experiment, in one group of the rats DIO was induced by providing ad libitum access to a pelleted high fat diet (60% kJ from fat; Diet 2127, Provimi Kliba AG, Kaiseraugst, Switzerland; energy content: 22 kJ/g, protein (w/w): 26%, carbohydrates: 1%, fat: 38.0%).

For the third experiment, as described in detail below, rats were given ad libitum access to a pelleted moderately high fat diet (31% kJ from fat; Diet D12266B, Research Diets Inc., New Brunswick; energy content: 18 kJ/g, protein: 21%, carbohydrates: 38%, 50% of which is sucrose, fat: 31%).

3.3 Amylin

Amylin (Bachem AG; Bubendorf, Switzerland) was diluted in 0.9% NaCl (Fresenius Kabi AG, Stans, Switzerland) in various concentrations, depending on the experiment (see below).

3.4 Feeding test procedure

In the first experiment, food was provided in internal, open food cups. In experiments two and three, food was offered in external food hoppers. For all experiments, food was weighed at least four to five times per week (to the nearest 0.1 gram) immediately before dark onset.

Before each test procedure, the animals were fed ad libitum, unless otherwise indicated, with their specified diet. With the exception of the experiment where animals underwent a prefeeding phase (see below), food was removed one hour before the start of the experiment. With the exception of the c-Fos study (Experiment 3), food was given back immediately after treatment and energy intake was measurement as described below.

All feeding trials were performed in a crossover manner so that each animal received all treatments. As such, each animal acted as its own control. For each individual crossover study, the two trials were performed 2 to 3 days apart. Time between trials allowed for animal recovery and for the clearance of exogenously administered substances.

3.5 Preparation of Hepatic Portal Vein Catheters

To produce the neck piece of the catheter, a metal adaptor was constructed from a 22G needle that was cut (about 2.5cm in length), the ends smoothed, and then bent in a 45-50° angle. A piece of silastic tubing (~ 5mm long; ID 0.508 x OD 0.94mm; Connectors, Tagelswangen, Switzerland) was placed in ether (5-7 ppm BHT, Hänseler AG, Herisau, Switzerland) for approximately 30 seconds and was fitted over the metal adaptor. Silastic tubing of the same diameter was cut to 25cm to create the intra-venous catheter, which was also placed in ether and was slipped over the metal adaptor. To further secure the catheter to the metal adaptor, a third piece of the same tubing (~ 5mm long) was placed over the catheter. Finally, a small piece (~ 1cm) of protective tubing (Silastic Tubings ID 0.762 x OD 1.651mm; Connectors, Tagelswangen, Switzerland) was placed in ether for 30 seconds and slipped over the catheter.

This protective tubing extended beyond the metal adaptor over the catheter tubing, and was intended to prevent strain on the catheter. Where the tubing was secured to the metal adaptor, a piece of surgical mesh (2x3cm; Bard Mesh; Davol Inc, Cranston, USA) was adhered with silk suture (Silkam USP 4/0, B.Braun Surgical GmbH, Melsungen, Germany), to enable adhesion to the skin.

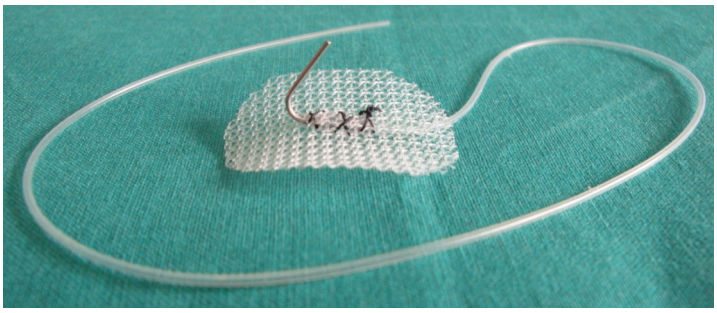

Figure 3.1: Hepatic Portal Vein Catheter

Prior to surgery, the adapter and catheter tubing were sterilzed in Kodan forte (Schülke & Mayr GmbH, Norderstedt, Germany) for 30min and then rinsed well with sterile 0.9% NaCl (Fresenius Kabi AG, Stans, Switzerland).

3.6 Osmotic Minipumps

Amylin was chronically administered peripherally by implanting osmotic minipumps (Alzet®, Model 2001, Durect Corporation, Cupertino USA) subcutaneously to investigate whether different levels of baseline amylinemia affect the anorectic action of an acute subcutaneous amylin injection. Minipumps with a mean pumping rate of 1.0µl per hour for seven days were used. On the morning of implantation, the minipumps were filled under sterile conditions with saline or amylin (total volume: 238µl/minipump, in concentrations described below).

3.7 Surgical Procedures

3.7.1 Hepatic portal vein (HPV) cannulation

The rats were fitted with intravenous catheters in the hepatic portal vein, to allow frequent and stress free blood sampling. One day before surgery the animals were treated with the antibiotic enrofloxacin (10mg/kg p.o., Baytril®, Bayer AG, Zürich, Switzerland). During surgery, the rats were placed on a heating pad to maintain body temperature and the eyes were

protected with an ophthalmic ointment (Vitamin-A Dispersa®, CIBA Vision AG, Niederwangen, Switzerland). The surgery was conducted under general anesthesia consisting of ketamine (75mg/kg i.p., Narketan®10, Vétoquinol AG, Ittigen Switzerland) and xylazine (10mg/kg i.p., Rompun® 2%,Bayer, Provet AG Lyssach). Prior to surgical intervention, the area between the scapulas and the abdomen was shaved and the skin was disinfected with Betadine® (Provet AG, Lyssach, Switzerland).

The catheter neck piece connector was subcutaneously fixed at the neck with bard mesh and exteriorized between the scapulae. The tip of the catheter was subcutaneously tunneled from the neck to a midline laparotomy, and then into the abdominal cavity. The intestines were gently removed from the abdominal cavity and arranged to expose the portal and mesenteric veins. Following ligation of afferent branches of the mesenteric vein with silk suture (Silkam USP 4/0, B.Braun Surgical GmbH, Melsungen, Germany), the wall of the mesenteric vein was penetrated with a 20G needle. The catheter was advanced through the mesenteric vein, with the tip placement visually confirmed in the hepatic portal vein just distal to the gastroduodenal vein. The catheter was then fixed with silk suture to adipose tissue adjacent to the insertion site, and blood flow in the catheter was confirmed.

The wound was closed with three lines of suture. The abdominal wall was closed with a line of simple running sutures (3-0 Vicryl), and the skin wound closed with a line of intracutaneous running sutures, followed by a line of interrupted horizontal mattress sutures (4-0 Vicryl). Post-operatively, rats were kept under a heat lamp until recovery from anesthesia, and received injections of buprenorphin (0.05mg/kg s.c., Temgesic®, Essex Chemie AG, Uznach, Switzerland), enrofloxacin (10mg/kg s.c., Baytril®) and 0.9% NaCl (5ml/rat s.c., Fresinus Kabi AG, Stans, Switzerland). Rats were treated with Baytril® p.o., and in some cases 0.9% NaCl s.c., for two days post-operation. During the recovery phase, the catheters were flushed every day with 200µl heparinized NaCl solution.

3.7.2 Osmotic minipump implantation

Rats were initially anesthetized by inhalation of 5% isoflurane (IsoFlo®, Provet AG Lyssach, Switzerland), then maintained on 2-3% isoflurane and placed on a heating pad to maintain body temperature during surgery. An ophthalmic ointment (Vitamin-A Dispersa®, CIBA Vision AG) was applied to protect the eyes. At the site of implantation, the rats were shaved

and the skin was disinfected with Betadine® (Provet AG). Under sterile conditions, a small incision was made between the scapulae and the minipump was subcutaneously implanted. The wound was closed with interrupted cutaneous sutures.

3.8 Blood Sampling

3.8.1 Sublingual vein sampling

Prior to blood sampling, rats were food deprived for 6 hrs. Immediately after being briefly anesthetized by inhalation of 5% isoflurane, rats were placed in a supine position and the tongue was extended from the mouth using a cotton-tipped applicator. One of the sublingual veins was punctured with a 20G needle, and blood was collected in a 500µl serum tube (Microvette®, SARSTEDT, Nümbrecht, Germany) and mixed with 5µl of a Protease Inhibitor Cocktail (P2714, Sigma, Missouri, USA). The blood remained at room temperature for at least 30 minutes and was then centrifuged for 10 minutes at 2500g. Serum was transferred to clean tubes and stored at - 20°C until use.

3.8.2 Portal vein sampling

Five to seven days before blood collection, rats were acclimated to the plexi-glass sampling chamber and tether, to reduce anxiety and activity on the day of collection. Prior to sampling, animals were food deprived for 12-14hrs. On the morning of the sampling, the lines were tested for patency and flushed with sterile NaCl. Approximately 45 min before dark onset, the rats were placed in the prepared sampling chambers and attached to the tethers to allow remote sampling in freely-moving rats. The tethers consisted of approximately 60 cm of polyethylene tubing (0.58mm ID, 0.96mm OD, Smith Medical International Ltd, Kent, UK) connected to the exteriorized catheter with a 22G stainless-steel coupler, protected with a spring (d 0.40 x D2.0mm, Kubo Tech AG, Effretikon, Switzerland) and suspended from an elastic line to allow the animals to move freely. Once the rats were attached, the catheters were flushed with about 50µl sterile NaCl every 5 minutes. The first baseline sample was collected after dark onset and before the rat was given access to food. Upon collection of the first sample, the rat was provided with a 40kJ pellet of food, either chow (3g) or high fat (2.2g). Samples were then collected at 7, 14, 21, and 28 min after the initiation of food intake. All samples were collected in 200µl EDTA-coated tubes (Microvette®, SARSTEDT, Nümbrecht, Germany), and mixed with 2µl of a Protease Inhibitor Cocktail. Samples were

centrifuged for 10 minutes at 2400g. Plasma was then transferred to clean tubes and stored at - 20°C until use. After the sampling procedure, the short catheter line was flushed with heparinized NaCl (200µl) and rats were given free access to food.

3.9 Perfusion and Tissue Proccessing

To analyze amylin induced c-Fos expression, rats were injected subcutaneously either with amylin (5µg/kg) or saline as a control. 120 minutes after this treatment, the rats were deeply anesthetized (Pentobarbital, 80mg/kg, i.p.) and transcardially perfused with phosphate buffer (PB 0.1M, pH 7.4) followed by 4% paraformaldehyde.

Following perfusion, the brain was removed and post fixed in 4% paraformaldehyde solution for two hours. Brains were then incubated for 48 hours at 4°C in 20% sucrose PB solution, frozen in chilled hexane, and stored at -20°. Using a cryostat (Leica CM3050S, Nussloch, Germany), coronal 20µm-thick sections were thaw mounted onto adhesion glass slides (Superfrost® Plus, Gerhard Menzel GmbH, Braunschweig, Germany) and stained for c-Fos immunoreactivity.

After air drying for 1hr at room temperature and rinsing in phosphate-buffered saline (pH 7.4) containing 0.1% Triton X-100 (PBST), the sections were incubated in normal goat serum (1.5% in 0.3% PBST; Jackson ImmunoResearch Inc, UK) for 2 hr, followed by 48 hr incubation in polyclonal rabbit anti-c-Fos (1:5K in 0.3% PBST; AB-5, Oncogene) at 4^{o}C. Sections were then incubated in biotinylated goat anti-rabbit IgG (1:400 in 0.3% PBST; Jackson ImmunoResearch Inc, UK) for 90 min at room temperature, rinsed in 0.1% PBST, and incubated for 1hr in avidin-biotin-peroxidase complex (1:100 in 0.3% PBST; Vectastain ABC kit, Vector). c-Fos was visualized by incubation for 4-5 min in nickel-cobalt enhanced DAB solution in PBS (0.04% DAB, 0.008% H_2O_2, 0.04% $NiCl_2$, and 0.08% $CoCl_2$). The slides were rinsed in PBS, dehydrated in graded alcohol, immersed in xylol, and cover-slipped with Entellan (Merck, Germany). In the area postrema, c-Fos positive cells were counted manually for each animal in a blind fashion under a light microscope (Axioscop 2, Zeiss).

3.10 Hormone Measurements

Circulating levels of plasma and serum amylin, insulin and leptin were measured using the Rat Endocrine Panel Milliplex MAP kits from Millipore (Millipore Corporation, Billerica, MA, U.S.A.). The assay is based on conventional sandwich assay technology and permits simultaneous detection of multiple hormones from a single sample. Briefly, according to the manufacturer's protocol, the microspheres were incubated with standards, controls, and the plasma/serum samples (10µl) in a 96-well microtiter filter plate on a plate shaker overnight (16-18 hr) at 2-8°C. After incubation, the plate was washed three times with an assay buffer (200µl/well) to remove excess reagents (by vacuum between each steps), followed by the addition of the detection antibody (50µl/well). Then the plate was placed again on a plate shaker for 60 min at room temperature, after which 50µl streptavidin-phycoerythrin was added to each well containing 50µl of detection antibody cocktail and incubated for an additional 30 min. After a final washing step (3 rounds of washing and fluid removal by vacuum filtration), the beads were re-suspended in buffer (100µl of Sheath Fluid) and the plate was analyzed using the Bio-Plex analyzer powered by xMAP Luminex technology (Bio-Rad Laboratories, Inc.) to determine the concentration of the hormones of interest. Data were analyzed by Bio-Plex Manager™ software versions 4.0 and 5.0 (Bio-Rad Laboratories, Inc.)

3.11 Body Composition Analysis

Body adiposity was determined by computerized tomography (CT) using the La Theta LCT-100 (Aloka; as described in detail in a recent paper from Jacquelien J.G. Hillebrand (Hillebrand et al., 2010)). The X-ray source tube voltage was set at 50 kV with a constant 1 mA current. The frozen carcasses were placed supine in the holders with an inner diameter of 120mm. First, a sagittal image of the entire animal was made to ensure proper placement in the holder and to set the scan area. Abdominal scans were done between vertebrae L1 and L6. Aloka software was used to estimate the volumes of adipose tissue, bone, air, and remaining tissue, using differences in X-ray density. Visceral (intraabdominal) and subcutaneous adipose tissues were distinguished based on the detection of the abdominal muscle layers; in some cases, this automated classification required manual image-by-image correction. Adipose tissue weights were then computed using the commonly used density factor of 0.92 g/cm^3. Total adiposity was calculated as the sum of visceral (intraabdominal) and subcutaneous fat mass.

3.12 Statistical Analysis

All data are expressed as mean ± SEM. Experiments performed in a crossover manner were analyzed using paired t-test, when appropriate. In experiments comparing independent groups, an unpaired t-test was used to detect statistically significant differences between the control saline treated and the amylin treated groups. When more than two groups were compared, data were analyzed using a two-way ANOVA, with Bonferroni's post-hoc test used to determine differences between individual groups. A p-value <0.05 was considered to be statistically significant. Statistical analyses were performed used GraphPad Prism (version 5.0, San Diego, CA, USA).

3.13 Description of individual experiments

3.13.1 Experiment 1

Does body composition influence the anorectic effect of amylin?

The aim of the first study was to assess if the acute anorectic response to amylin is altered in rats chronically fed a high fat diet or being food restricted, to achieve a state of diet induced obesity or chronic underfeeding, respectively.

The timeline of Experiment 1 is shown in Figure 3.1. After the acclimation phase, the animals were divided into three randomized groups (7-8 rats/group). The control group was fed ad libitum with standard chow diet. A second group was fed ad libitum with a HF diet containing 60%kJ from fat to achieve DIO. A third group was maintained on chow and restricted to 80% of the ad libitum intake of the chow-fed controls for 11 weeks (approximately 20g of food given daily at dark onset), and then switched to the HF diet for the remainder of the experiment (i.e. for 3 weeks).

Over the course of the 14-week experiment, 8 feeding trials were performed, each testing the anorectic response to different doses of amylin at various points of HF feeding or food restriction. The objective of each feeding trial is summarized in Table 3.1.

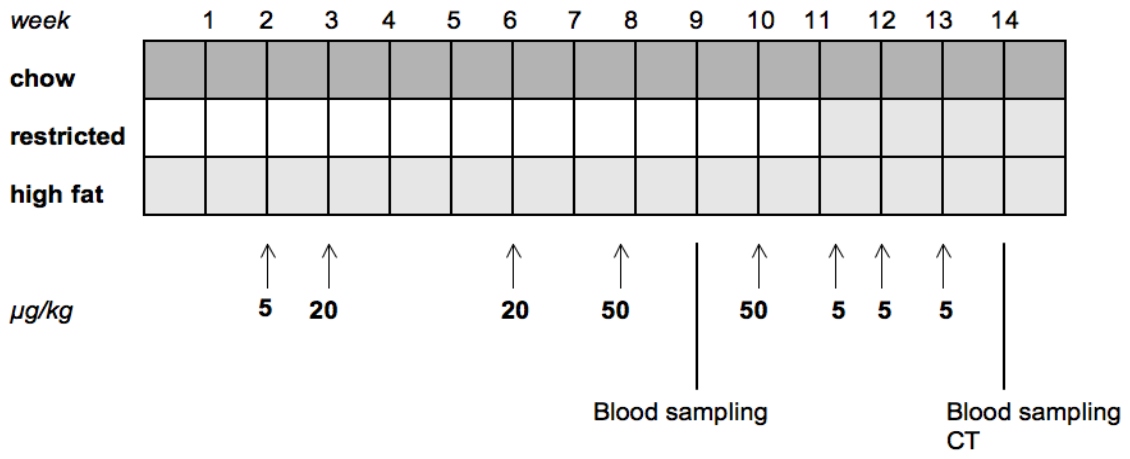

Figure 3.1 Timeline for Experiment 1. Arrows indicate feeding trials and the corresponding dose of amylin administered. (Dark grey: chow ad libitum; white: chow restricted; light grey: HF ad libitum)

For each feeding trial, amylin or saline was administered subcutaneously to non-fasted rats immediately before dark onset. Energy intake was measured 1, 2 and 4 hours following the treatment. For some trials (See Table 3.1), animals were given access to food for 60 min prior to the injection. In the feeding trial number 5, the amount of the food that was given to the restricted rats in the prefeeding phase was matched to the amount of food the chow control group ate in that time, and therefore were quasi pair-prefed to the control group.

Feeding Trial	Week Number	Amylin (µg/kg)	Objective
1	2	5	Does short-term HF- or restricted-feeding alter the anorectic response to a low dose of amylin?
2	3	20	Does short-term HF- or restricted-feeding alter the anorectic response to a moderate dose of amylin?
3	6	20	Can a moderate dose of amylin attenuate food intake when restricted rats are prefed with standard chow (132 kJ; 10g) 1hr before treatment?
4	8	50	Can a high dose of amylin attenuate food intake when restricted rats are prefed with standard chow (132 kJ; 10g) 1hr before treatment?
5	10	50	Can a high dose of amylin attenuate food intake when restricted rats are pair-prefed with standard chow (66 kJ; 5g) 1hr before treatment?
6	11	5	Following 3 days of HF refeeding, do the restricted rats respond to a low dose of amylin?
7	12	5	Following 1 week of HF refeeding, do the restricted rats respond to a low dose of amylin?
8	13	5	Following 2 weeks of HF refeeding, do the restricted rats respond to a low dose of amylin?

Table 3.1 Summary of feeding trial objectives

Sublingual blood samples for hormone measurements were collected during weeks 9 and 14. After week 14, rats were euthanized by an overdose of pentobarbital (300mg/kg i.p.), and frozen prior to performing the CT scans to determine body composition.

3.13.2 Experiment 2

Does chronic peripheral elevation of amylin levels induce a change in amylin sensitivity?

To investigate if elevated baseline levels of circulating amylin change the sensitivity to the acute anorectic action of amylin, circulating baseline amylin was clamped to different levels using osmotic minipumps. Two amylin doses (5 and 10µg/kg/day) were infused based upon circulating levels of DIO rats observed in Experiments 1 and 2, as well as from published work (Mack et al., 2007; Trevaskis et al., 2008), and compared to saline infused controls.

For the duration of the experiment, rats were allowed ad libitum access to standard pellet chow. The modified counterbalanced experiment was performed over three weeks, during which every animal received each combination of minipump infusate (saline, 5 or 10µg/kg/day amylin) and acute amylin injection (saline, 5 or 20µg/kg, s.c.). The minipumps were implanted on the Friday in the week before the respective feeding trials. The schedule of acute amylin administration is shown in Figure 3.2. For all feeding trials, saline or amylin was administered to non-fasted rats immediately before dark onset. Energy intake was measured 30, 60 and 120 min following the treatment.

Each week, following the completion of the third feeding trial and before the minipumps were exchanged, sublingual blood samples were taken to assess baseline circulating amylin levels.

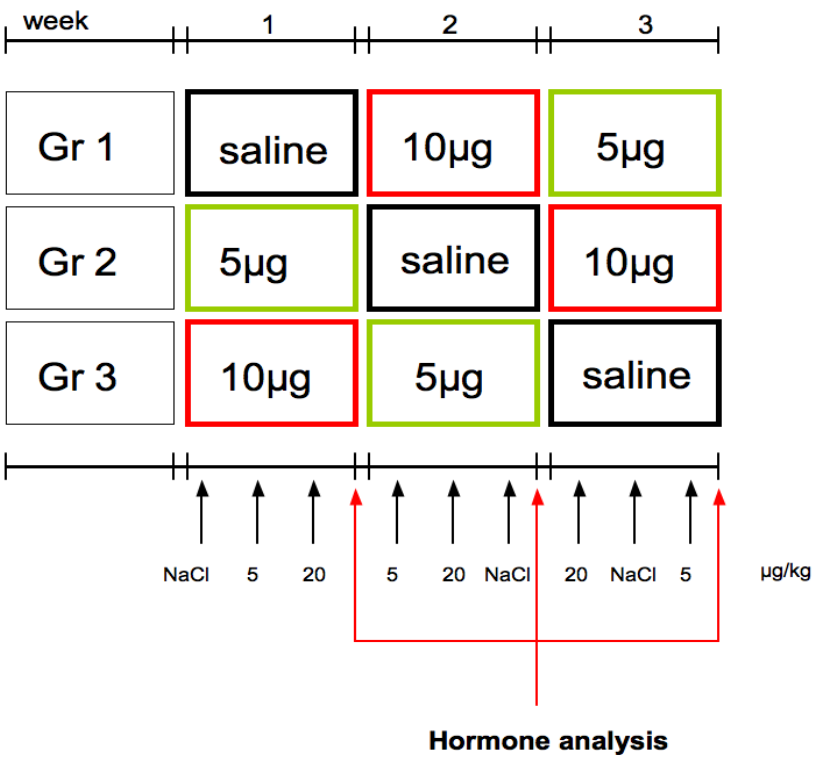

Figure 3.2 Schedule of acute amylin administration used in Experiment 2

3.13.3 Experiment 3

Is the amylin secretion pattern, as measured from the portal vein, after a test meal, altered in diet-induced obese rats?

Two studies were conducted to determine if diet-induced obesity (DIO) alters the pattern of meal-induced amylin secretion.

3.13.3.1 Experiment 3a

The timeline of Experiment 3a is shown in Figure 3.3. Following the acclimation time, sublingual blood samples were taken to measure baseline amylin, insulin and leptin levels. From this day, all the rats were given ad libitum access to a pelleted high fat diet (HF; 60%kJ from fat) for approximately 2 weeks. Then the rats were split into two groups. The half that gained the highest percentage of body weight on the HF diet was maintained on HF diet for the remainder of the experiment. The lower-gaining half was maintained on standard chow for the remainder of the experiment.

In the 5th week on the high fat diet (and three weeks after the switch to chow) all the animals were fitted with HPV catheters (Sx). After two weeks of recovery and adapting to the experimental sampling cages, blood samples were collected from the portal vein as described above.

Following conclusion of HPV sampling, the efficacy of amylin to reduce eating was tested in a single crossover feeding trial. Saline or amylin (5µg/kg, s.c.) was administered to non-fasted rats immediately before dark onset. Energy intake was measured 30, 60 and 120 min following the treatment.

Sublingual blood samples for hormone measurements were collecting during weeks 0, 2, 5 and 9 to assess baseline amylin, leptin, and insulin levels.

After week 9, rats were given a final injection of either saline or amylin (5µg/kg, s.c.). 120 minutes later, rats were anesthetized with pentobarbital (80mg/kg, i.p.) and transcardially perfused. Brains were then removed and processed for c-Fos immunhistochemistry as described above.

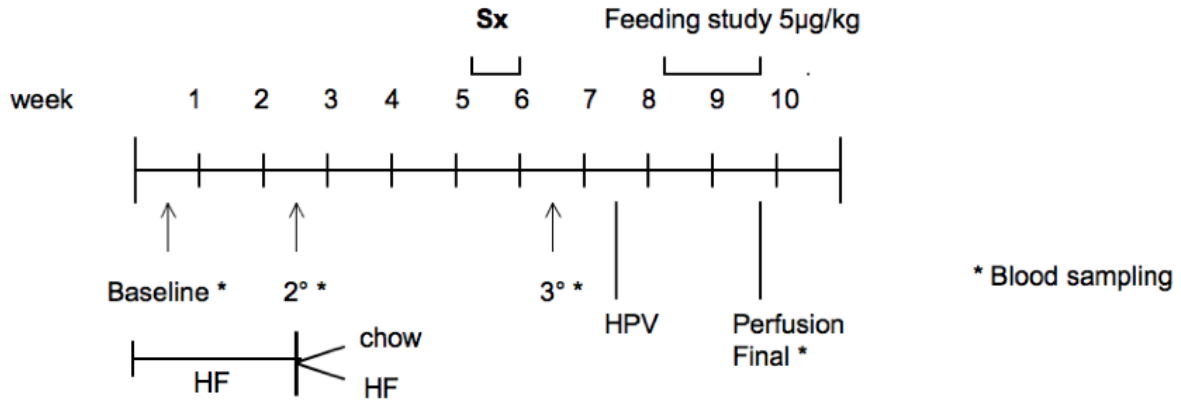

Figure 3.3 Timeline for Experiment 3a; for abbreviations see text.

3.13.3.2 Experiment 3b

To extend the findings from experiment 3a, we included an additional DIO-resistant (DR) control group in the second study. To achieve this, we made use of the fact that when outbred Sprague-Dawley rats are fed a high-fat diet, approximately half will become obese (DIO), while the other half remain relatively lean (DR); these DR rats gain as much weight as chow-fed controls (Levin et al., 1997). Because the DR rats do not gain excessive weight or fat mass when fed a high-fat diet, this group controls for the effects that the high-fat diet may have on meal-induced amylin secretion, independent becoming obese. As in the first study, the development of DIO was tracked throughout the experiment by way of hormone level determination.

The timeline of Experiment 3b is shown in Figure 3.4. Following acclimation time, sublingual blood samples were taken to measure baseline amylin, insulin, and leptin levels. From then, all the rats were given ad libitum access to a pelleted moderatly high fat diet (31% from fat) containing sucrose. After 2 weeks on this diet, body weight and growth patterns were assessed, and rats were separated into three groups using previously described methods (Levin et al., 1997). Briefly, the tertile gaining the highest percentage of body weight on the HF diet were designated the DIO-prone group of rats, and were maintained on HF diet for the remainder of the experiment. The middle tertile became the control group; these rats were maintained on standard chow for the remainder of the experiment. The lowest gaining tertile, was designated the DIO-resistant (DR) group of rats, and also was maintained on the HF diet for the remainder of the experiment.

In week 6, all the animals were fitted with HPV catheters (Sx). After one week of recovery and adapting to the experimental sampling cages, blood samples were collected from the portal vein as described above.

Following conclusion of the HPV sampling, the efficacy of amylin to reduce eating was tested in several acute feeding trials. All studies were performed in a crossover manner and doses increased from 5µg/kg, to 20µg/kg, to 50 µg/kg. Saline or amylin was administered to non-fasted rats immediately before dark onset. Energy intake was measured 30, 60 and 120 min following the treatment.

Sublingual blood samples for hormone measurements were collecting during weeks 0, 2, 5 and 13 to assess amylin, leptin, and insulin levels.

After week 13, rats were given a final injection of either saline or amylin (5µg/kg, s.c.). 120 minutes later, rats were anesthetized with pentobarbital (dose, i.p.) and transcardially perfused. Brains were then removed and processed for c-Fos immunhistochemistry as described above.

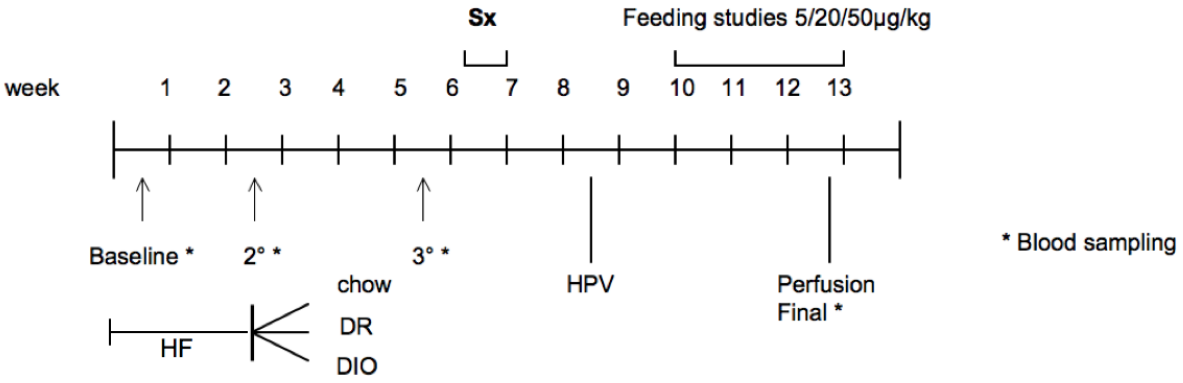

Figure 3.4 Timeline for Experiment 3b; for abbreviations see text.

4 Results

4.1 Obesity resulting from long-term maintenance on high fat diet attenuates the anorectic effect of amylin

Experiment 1

Baseline body weight was similar across groups (240-260g), with no significant differences at the start of the experiment. Figure 4.1 shows the average body weight of the three groups for the duration of the study. At the beginning of week 12 (day 77) , the restricted rats were switched to the high fat diet which was offered ad libitum for the remainder of the experiment.

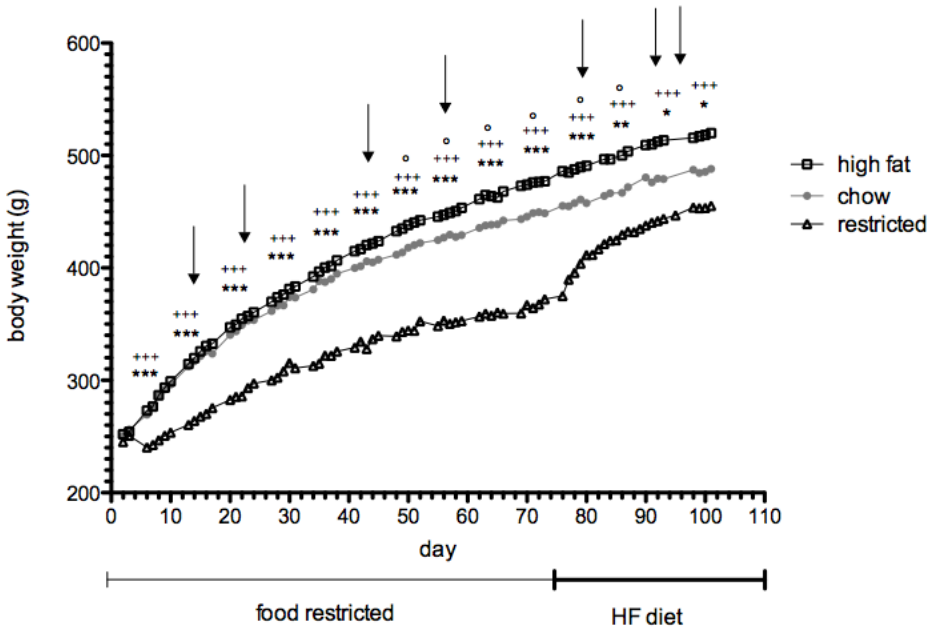

Figure 4.1: Mean body weight in rats maintained on a regime of chow (n=8) or high fat diet (n=8) for 14 weeks, or restricted (n=7) for 11 weeks (day 76) to 80% of ad libitum food intake and then switched to high fat diet for the 3 final weeks (days 77 to 101) of the experiment. Arrows indicate times at which feeding trials were performed. The different symbols denote significant differences among the three diet groups; * chow vs restricted, °chow vs high fat, + high fat vs restricted; *p<0.05, **p<0.01, ***p<0.001

The body weight of the restricted rats was significantly lower during the entire experiment compared to the other two groups. Body weight remained significantly lower during the high-

fat refeeding period of the restricted animals until the end of the observation period. When comparing the chow control group and the high fat fed animals, a significant difference was observed between weeks 6 and 12 on the different diets. During the last two weeks of the experiment the difference in body weight between these two diet groups was no longer significant.

Figure 4.2A shows the effect of acute peripheral amylin administration in rats maintained on the different feeding regimes for two weeks (day 14/16). The rats were injected in a crossover manner with either saline or 5µg/kg amylin. With the exception of the restricted group, amylin-treated rats showed a significant reduction in energy intake, as compared with the saline controls, at 60 and 120 min after injection; after 240 min, the difference between the amylin- and saline-treated animals was no more significant in any diet group. The average body weights of each group are shown in Figure 4.2B; there was a significant difference between the chow and the restricted and the high fat and the restricted groups, respectively, on the day of injection (Figure 4.2B). At this time point, the body weight between chow and high-fat rats did not differ.

A B

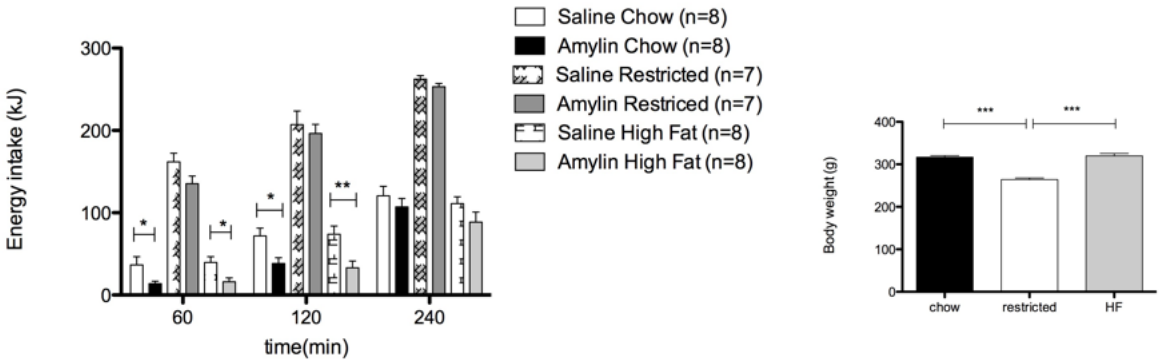

Figure 4.2: Mean (± SEM) cumulative energy intake 60, 120, and 240 minutes after saline or amylin (5µg/kg, s.c.) injection in rats maintained on chow, high fat diet, or 80% food restriction for 2 weeks (A), and the average body weight of each group at the time of injection (B). Symbols denote significant differences beween saline- and amylin-treated groups within the respective diet regimes (A) or between feeding groups (B); *p<0.05, **p<0.01, ***p<0.001

After three weeks on the feeding regimes, the rats were tested again for their response to a higher dose of amylin (20µg/kg). The effects on eating were similar in that amylin significantly reduced eating in chow and high-fat rats but not in restricted rats (4.3A). Similar to the two week time point, body weight was significantly lower in the restricted than in the chow or high fat rats (4.3B). In addition to what was previously observed in the 5µg/kg amylin trial, a significant reduction in energy intake in the chow-fed group was also detected four hours after treatment in the chow controls.

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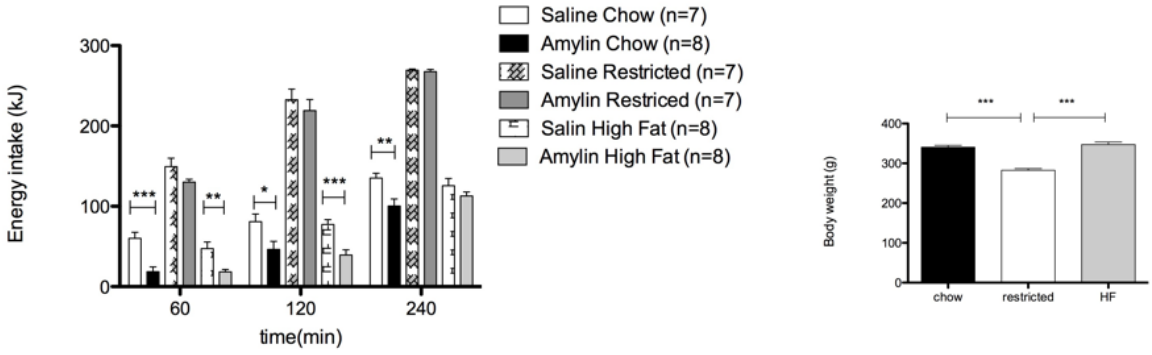

Figure 4.3: Mean (± SEM) cumulative energy intake 60, 120, and 240 minutes after saline or amylin (20µg/kg, s.c.) injection in rats maintained on chow, high fat diet, or 80% food restriction for 3 weeks (A) , and the average body weight of each group at the time of injection (B). Symbols denote significant differences beween saline- and amylin-treated groups within the respective diet regimes (A) or between feeding groups (B); *p<0.05, **p<0.01, ***p<0.001

It seemed that in the first two feeding trials, the increased drive to eat in the food-restricted group could not be suppressed by amylin. Because one aim of the experiment was to test if amylin efficacy is increased in rats of decreased body weight, the experimental design was changed for the subsequent studies. In the third through fifth feeding trials, all animals were allowed to eat one hour prior to the amylin injection, i.e. during the first hour of the dark phase. In trials 3 and 4, rats were provided with 132 kJ of food during this hour, which corresponds to approximately 50% of their daily intake (10g). In both trials the food-restricted group ate the entire 132 kJ in the hour before treatment. The chow controls and HF-fed ate on average 57 and 53 kJ, respectively. In trial 3, a 20µg/kg dose of amylin still did not

suppress food intake at 60 or 120 min after administration in the restricted group (data not shown). When the amylin dose was increased further in trial 4, i.e. when a relatively high dose of amylin (50µg/kg) was administered, the decrease in food intake in the food-restricted group induced by amylin was significant (See Figure 4.4A). On the day of the injection, there was a significant difference in body weight between the chow and the restricted, and the high fat and the restricted groups, respectively (Figure 4.4B).

In trial 5 (data not shown), in which all groups were pre-fed with an amount of food that all rats had eaten in the previous trials 3 and 4 (50 kJ) and despite given the relatively high dose of 50µg/kg amylin, the amylin effect disappeared; in other words, no significant decrease in food intake was observed in the food restricted group (data not shown). In all these experiments (trials 3-5), the chow- and HF-fed groups continued to show similar sensitivity to the anorectic effect of acute amylin administration.

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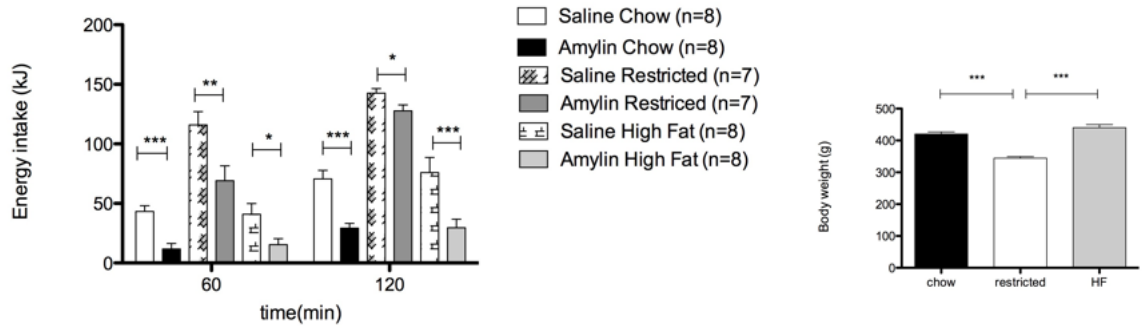

Figure 4.4: Mean (± SEM) cumulative energy intake 60 and 120 minutes after saline or amylin (50µg/kg, s.c.) injection in rats maintained on chow, high fat diet, or 80% food restriction for 8 weeks. Rats were prefed for 1hr with their specified diet before treatment. In this prefeeding hour, restricted rats ate the full preload of 132 kJ, whereas the chow controls and the HF-fed ate on average 57 and 53 kJ, respectively (A). Average body weight of each group at the time of injection (B). Symbols denote significant differences beween saline- and amylin-treated groups within the respective diet regimes; *p<0.05, **p<0.01, ***p<0.001

For the final three trials (trial 6-8) using these groups of rats, the restricted animals were refed ad libitum with the high fat diet. In trial 6, performed on days 3 and 4 following the switch to HF diet, a low dose of amylin (5µg/kg) significantly reduced food intake in the chow-fed group only (Figure 4.5A).

When the same experiment was repeated a few days later (trial 7), i.e. 1 week after the diet switch, in the previously restricted rats the outcome was similar (Figure 4.6A). It is important to note that average body weight was significantly different between all groups on these injection days (Figures 4.5B and 4.6B) i.e. body weight in the high fat group was significantly higher than in the chow group. Interestingly, the anorectic effect of amylin in the high fat group began to dissipate after prolonged intake of HF diet, in this case after approximately 12 weeks on the HF diet. In other words, a significant decrease in energy intake in the HF group could only be detected two hours after amylin treatment, while the chow control group showed a significant decrease at all time points.

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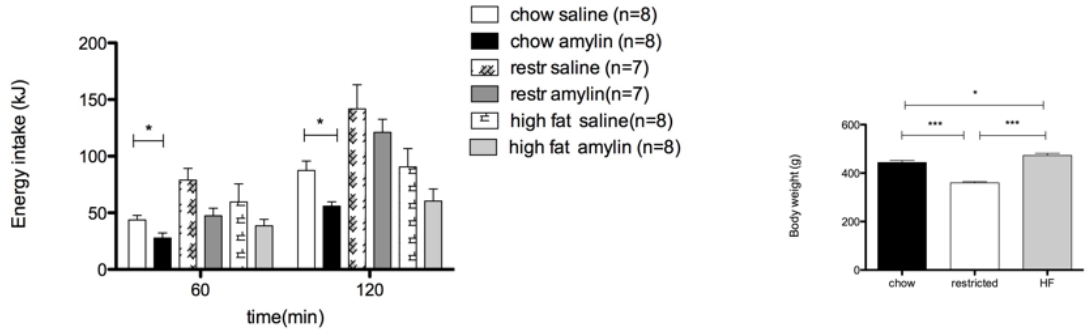


Figure 4.5: Mean (± SEM) cumulative energy intake 60 and 120 minutes after saline or amylin (5µg/kg, s.c.) injection in rats maintained on chow or high fat diet. The chow controls and the HF-fed rats had been on their respective diets for 11 weeks, the previously-restricted rats had been switched to HF 3 days before injection (A). Average body weight of each group at the time of injection (B). Symbols denote significant differences beween saline- and amylin-treated groups within the respective diet regimes; *p<0.05, **p<0.01, ***p<0.001

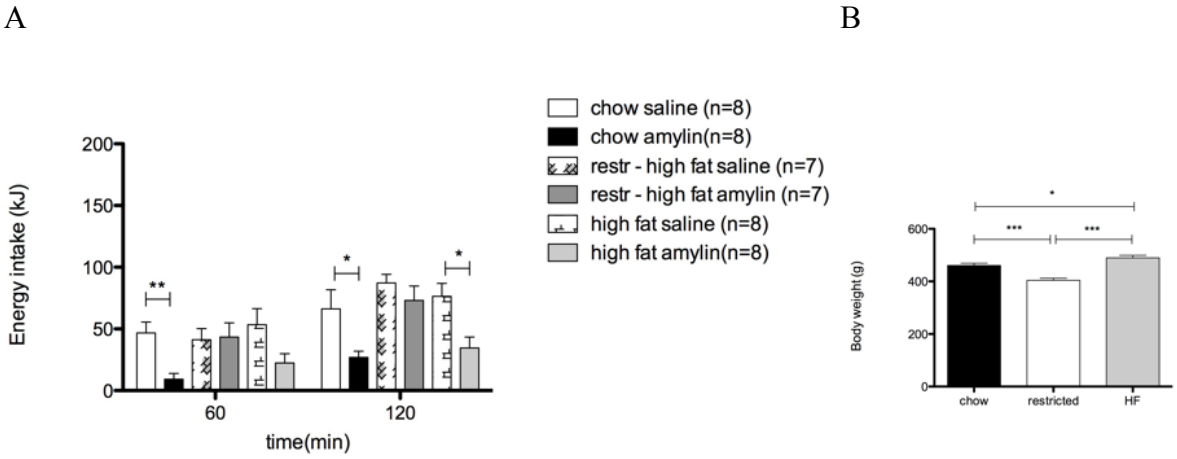
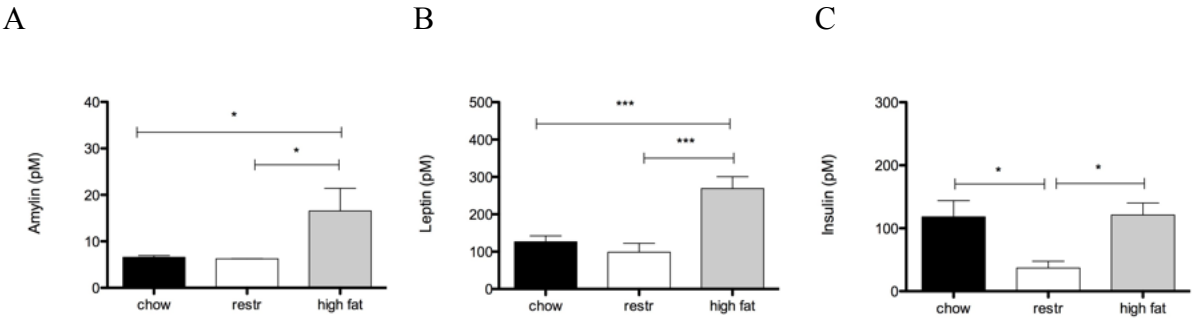


Figure 4.6: Mean (± SEM) cumulative energy intake 60 and 120 minutes after saline or amylin (5µg/kg, s.c.) injection in rats maintained on chow or high fat diet. The chow controls and the HF-fed rats had been on the diet for 12 weeks, the previously-restricted rats had been switched to HF 1 week before injection (A). Average body weight of each group at the time of injection (B). Symbols denote significant differences beween saline- and amylin-treated groups within the respective diet regimes; *p<0.05, **p<0.01, ***p<0.001

Figure 4.7 shows baseline concentrations of amylin, leptin, and insulin in fasted rats measured in weeks 9 and 14. In week 9, rats fed the high fat diet demonstrated significantly elevated levels of amylin and leptin (Fig. 4.7A and B), when compared to chow-fed or food-restricted rats. Chow fed and restricted rats did not differ even though restricted rats weighed significantly less. Furthermore, insulin levels were significantly higher in high fat- and chow-fed rats compared to restricted rats (Fig. 4.7C). From the terminal blood samples collected during week 14 (Fig. 4.7D - F; note that the previously restricted rats had been switched to high fat diet 3 weeks earlier), the differences in amylin and insulin levels were no longer detectable, despite a significant difference in body weight between formerly restricted and chow-fed or high fat-fed rats, respectively. Of note, all values were higher than in week 9. At this time point, a significant increase in circulating leptin was observed in the high fat group compared to the chow controls (Fig. 4.7E). Interestingly and despite the significant difference in body weight, there was no significant difference in leptin levels between the high fat group, which had been maintained on HF diet for 14 weeks, and the previously-restricted group, which had only been on HF diet for 3 weeks.

Week 9



Week 14

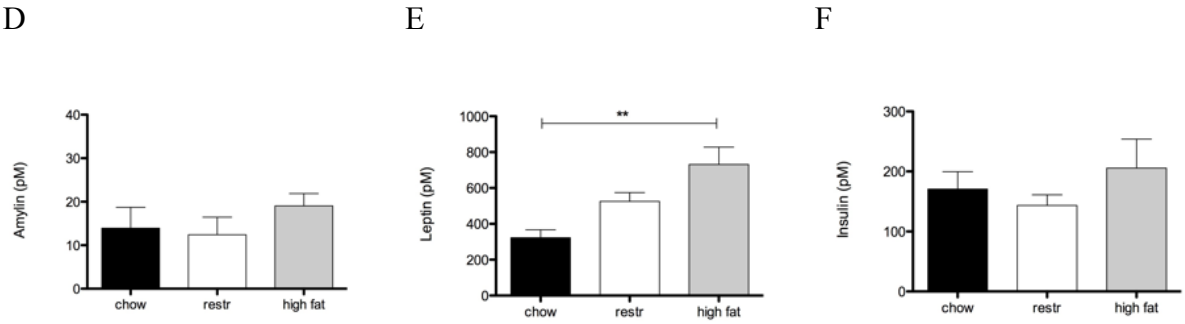


Figure 4.7: Effect of different diets on mean (± SEM) amylin, leptin ans insulin levels, in rats maintained on f chow (n=8), high fat diet (n=7), or 80% food restriction (n=8) for 9 (A-C) and 14 (D-F) weeks. In Figures D-F, previously restricted rats had been swiched to HF-diet for 3 weeks. Symbols denote significant differences between the diet regimes; *p<0.05, **p<0.01, ***p<0.001

Average body composition of the three groups at the time of sacrifice (week 14), as determined by CT scan, is shown in Figure 4.8. For total body fat mass (Figure 4.8A) there was no significant difference between the high fat and the previously-restricted group, but both groups had significantly higher total body fat than the chow-fed controls. Visceral fat mass (Figure 4.8B) was significantly higher in high fat-fed rats compared to the other two groups. Similar to total body fat mass, the previously-restricted rats showed significantly higher values for subcutaneous fat than chow-fed rats. Again, no difference was detected between the high fat and the previously-restricted group in the subcutaneous fat mass (Figure 4.8C), but both groups had significantly higher levels compared to the chow-fed group.

A B C

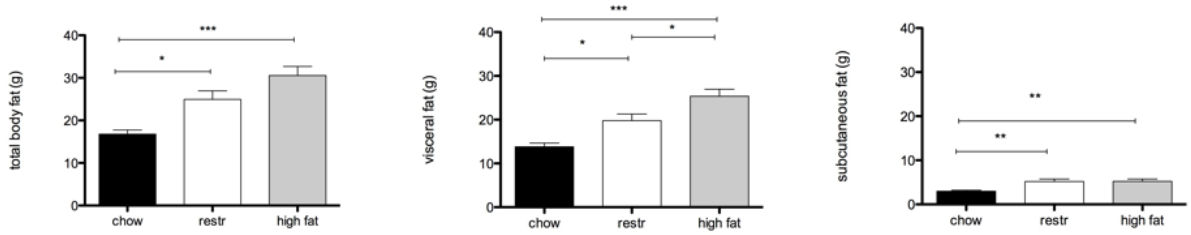


Figure 4.8: Effect of different diets on mean (± SEM) total (A), visceral (B) and subcutaneous (C) body fat in rats maintained on chow (n=8), high fat diet (n=7), or 80% prior food restriction (n=8) for 14 weeks. Previously restricted rats had been swiched to HF-diet for 3 weeks. Symbols denote significant differences beween the diet regimes; *p<0.05, **p<0.01, ***p<0.001

4.2 Hyperamylinemia alone does not reduce the sensitivity to an acute amylin injection

Experiment 2

Figure 4.9 shows the percentage of body weight gain under the influence of two doses of chronic amylin treatment (5 and 10µg/kg/day) compared to control animals receiving saline and compared to the respective baseline body weight (100%) at the beginning of the 1-week infusion. Although there were no significant differences among the three groups, control animals gained slightly more weight than the amylin treated rats. Hence, as expected, there was a tendency that body weight gain was inversely correlated to the amylin concentration.

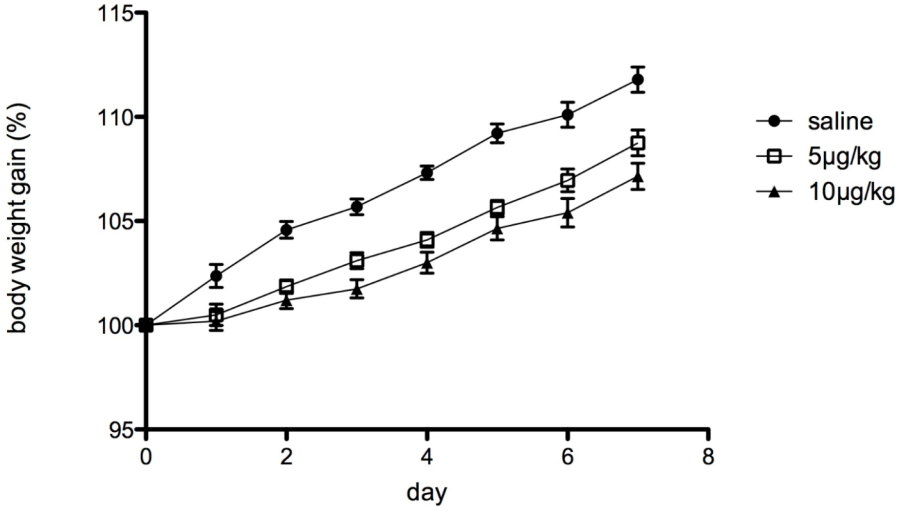


Figure 4.9: Mean body weight gain (in %) in rats (n=20) implanted with osmotic minipumps delivering either NaCl in the control group, or two doses of amylin (5µg/kg/day or 10µg/kg/day). All data across the 3 week experiment are compiled.

Correspondingly, daily energy intake data (Figure 4.10) show that the amylin-treated rats demonstrated some decrease in energy intake compared to the control group, even though there were no significant differences among groups on any day or in total cumulative energy intake after each week.

A B

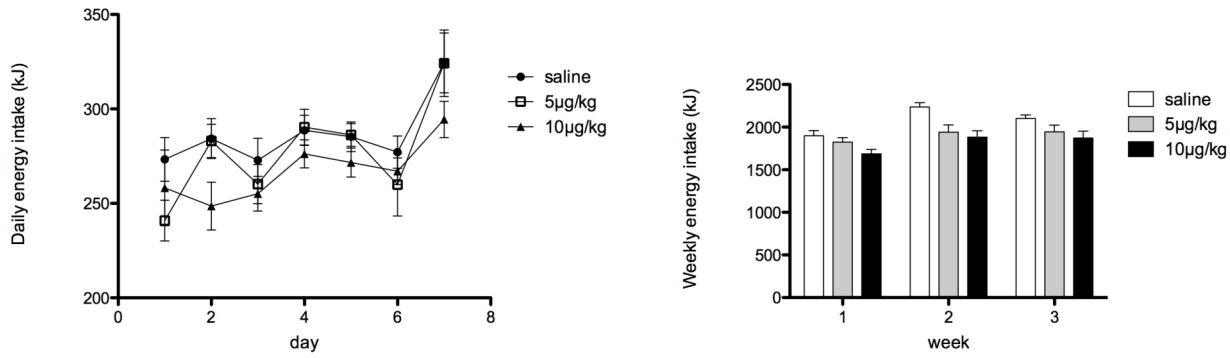


Figure 4.10: Mean (± SEM) daily (A) and weekly (B) energy intake in rats (n=20) implanted with osmotic minipumps delivering either saline in the control group, or amylin (5 or 10µg/kg/day). Compiled data of baseline food intake during the 3 weeks of infusion.

Figure 4.11 shows the levels of baseline circulating amylin as measured at the end of the respective infusion week in ad libitum fed rats. When data from the three weeks are compiled, we observed significantly higher circulating amylin concentrations in animals that received chronic amylin at a dose of 5 or 10 µg/kg/day.

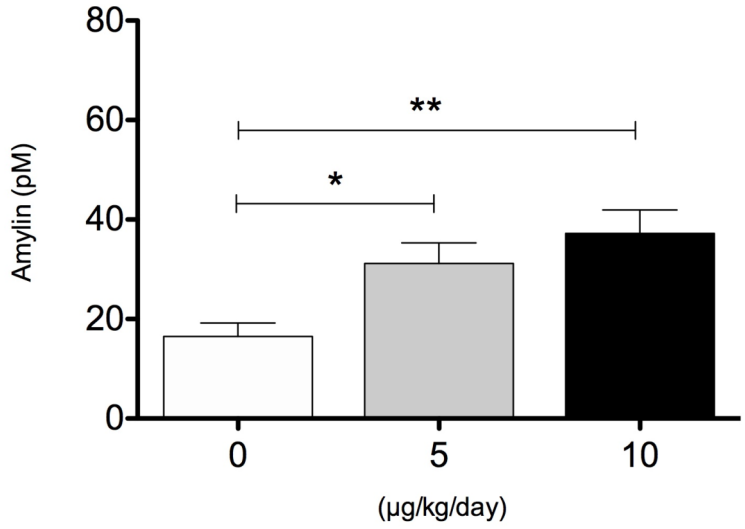


Figure 4.11: Mean (± SEM) combined amylin levels in rats (n=20) implanted with minipumps delivering either saline, 5 or 10 µg/kg/24h for one week. Symbols denote significant differences beween the diet regimes; *p<0.05, **p<0.01, ***p<0.001

Figure 4.12 shows the effect of acute peripheral amylin on energy intake in rats with differing baseline amylin concentrations but relatively similar body weight. We saw no difference in the anorectic effect of acute amylin administration dependent on the baseline amylin levels. Across all groups, the higher the dose of the acute amylin treatment, the larger the anorectic effect of amylin.

More precisely, at all timepoints and in all groups, energy intake was significantly lower following an acute injection of 20µg/kg amylin than in the saline-injected control group. In rats with the 5µg/kg/day-minipump, we also saw a significant decrease in energy intake after acute amylin (5µg/kg) at 30 min post-treatment. The main difference post amylin treatment was observed after two hours, where 5µg/kg amylin significantly decreased food intake in the control group, whereas we did not observe a significant effect in the 5µg- and the 10µg/kg/day-minipump group after an acute treatment with 5µg/kg of amylin.

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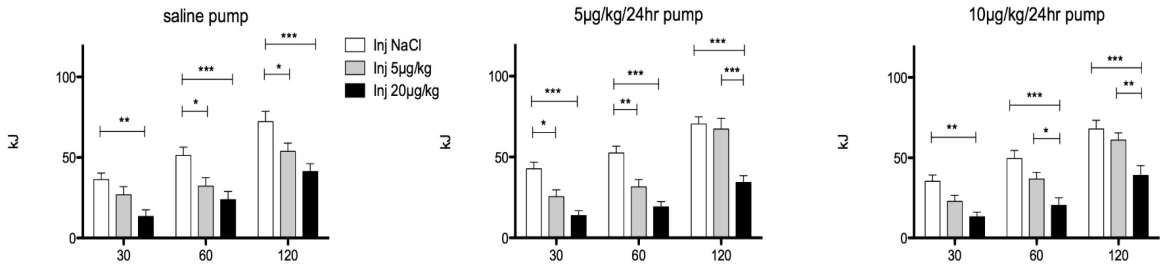

Figure 4.11: Mean (± SEM) cumulative energy intake 30, 60 and 120 minutes after saline, amylin (5 µg/kg, s.c.) or amylin (20µg/kg, s.c.) injection in rats implanted with a osmotic minipump delivering either saline (A), amylin (5µg/kg/day; B) or amylin (10µg/kg/day; C) (n=6-7). Symbols denote significant differences beween saline- and amylin-treated groups; *p<0.05, **p<0.01, ***p<0.001

4.3 Meal-induced amylin secretion in lean and obese rats

The final experiment focused on the pattern of meal induced amylin and insulin release from the pancreas and the resulting blood levels in the hepatic portal vein in obese and non-obese rats. For this experiment, we used either a high fat diet with 60%kJ of fat to induce obesity (experiment 3a) or the high fat diet used in experiment 3b with 30%kJ from fat and with a sucrose component to develop DIO and DR rats according to the bimodal pattern of inheritance observed in Sprague Dawley rats (Levin et al., 1997) (experiment 3b).

4.3.1 Experiment 3a

Figure 4.13 shows the meal-induced pattern of amylin and insulin secretion from individual rats maintained on the high fat diet for approximately 7 weeks. Each rat was tested twice with 1 day between tests. At time point 0, all animals were provided with an initial meal (40kJ, corresponding to approximately 9% of average daily food intake) of high fat diet subsequent to 12 hours of fasting. This amount of food was eaten within approximately two minutes. Within 7 to 8 minutes after meal initiation amylin and insulin levels increased 2 to 3 times compared with baseline levels. 14 minutes after the start of the meal, amylin and insulin returned to baseline levels. There seemed to be a second increase during the next 14-15 min, although this rise was smaller and slower than the first one. Due to the small number of animals in this experiment and the lack of blood samples from the chow control group,

proper statistical analysis of the data was not performed and the results were not compared to a control group.

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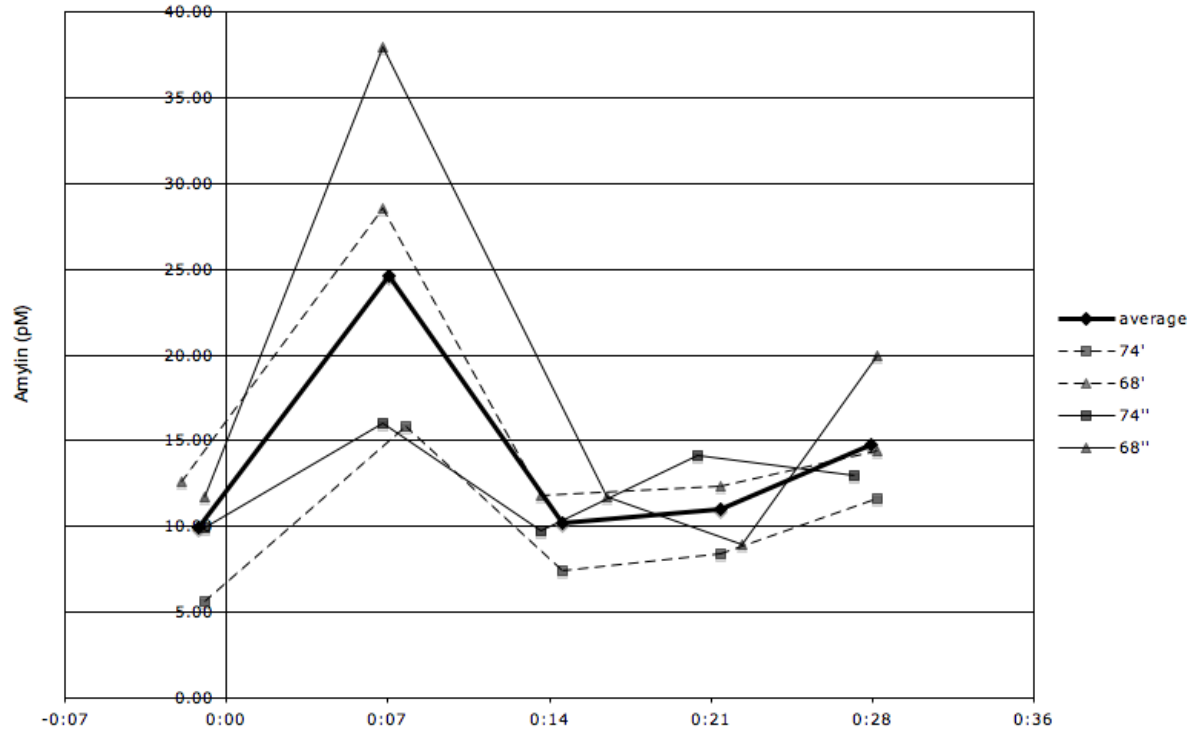

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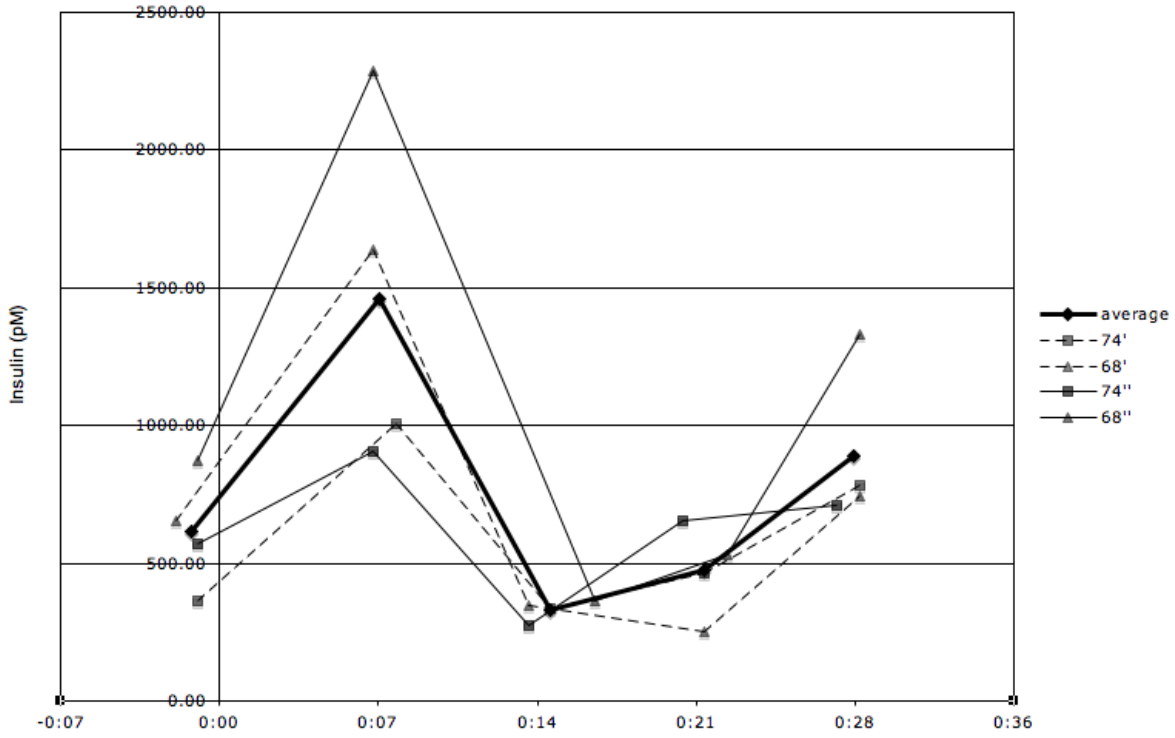

Figure 4.13: Amylin (A) and insulin (B) concentration in the hepatic portal vein in individual obese rats (n=2) and the average of all animals combined after a 40 kJ test meal at time point 0:00:00.

Figure 4.14 shows circulating hormone levels at various time points over the course of the experiment. The two-way ANOVA detected significant effects of time and of the diet group. The effect over time for amylin (p=0.0035), insulin (p=<0.0001) and leptin (p=<0.0001) and between the diet groups for amylin (p=0.0106), insulin (p=<0.0001) and leptin (p=<0.0001) were significant. Comparing the two diet groups, the high fat-fed animals showed significantly higher insulin levels than the control group at weeks 5 and 9. The difference between leptin levels reached significance at week 9.

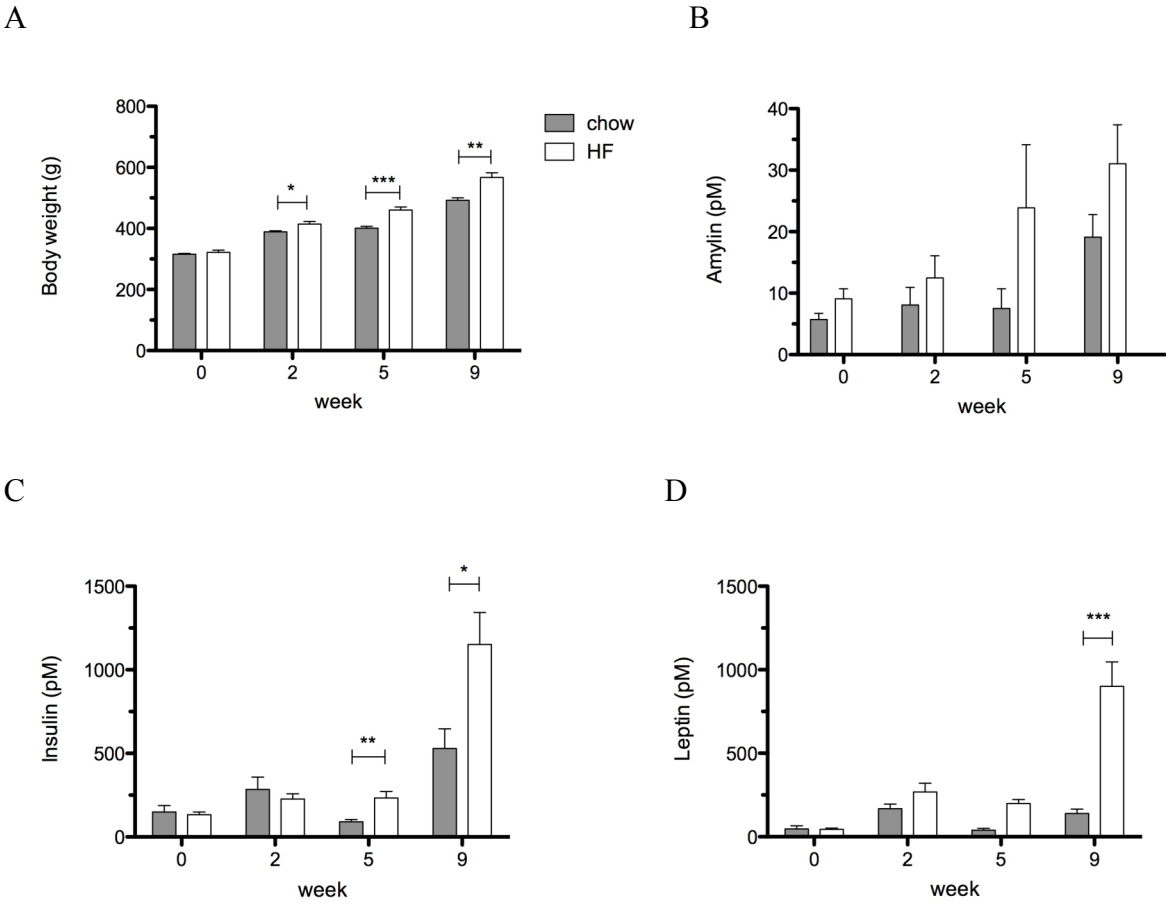

Figure 4.14: Average (± SEM) body weight of each group at various time points after exposure to the high fat diet (n=6-7) compared to chow (n=6-8) (A) and mean (± SEM) amylin (B), leptin (C) and insulin (D) levels in rats maintained on of chow or high fat diet for 0, 2, 5 and 9 weeks. Symbols denote significant differences beween the diet regimes at individual time points; *p<0.05, **p<0.01, ***p<0.001

4.3.2 Experiment 3b

Figure 4.15 and 4.16 show the pattern of amylin (Figures 4.15 A–C) and insulin (Figures 4.16 A-C) hepatic portal vein concentration in animals fed a test meal of either standard chow or a high fat diet, after maintenance on the respective diets for approximately eight weeks. Rats were grouped into DIO and DR according to Levin (Levin et al., 1997); these groups were compared to chow controls. At the time of sampling, chow animals weighed on average 519g, DIO animals 545g an DR animals 496g; DIO animals had significantly higher body weights compared to chow control and DR animals.

In all three groups we observed a high variability between individual animals; further we were not able to collect blood samples at all timepoints from each animal. Figures 4.15D and 4.16D show the average concentrations of amylin and insulin of each diet group plotted on a single graph. The average amylin secretion pattern suggests that there is a tendency for baseline amylin levels in the DIO and DR groups to be higher than in the chow group. Furthermore, we observed signficantly higher postprandial amylin levels in the DIO and DR group compared to the chow group, in particular at the second sampling timepoint 7 minutes after the onset of the test meal. Even though DIO rats were significantly heavier than the DR cohort at the time of sampling, both groups had been maintained on the same high fat diet for the same duration; the high fat diet was also consumed during the test meal. Thus, it seems that the higher and faster meal induced increase in amylin levels in the DIO and DR animals is more likely due to the high fat diet itself then due the higher body weight of DIO.

We also observed some differences in the secretion pattern of insulin, compared to amylin, across the three groups. The baseline levels of insulin did not differ among the three groups. We did, however, observe significantly higher postprandial insulin levels in the DIO and the chow group, compared to the DR group, 14 minutes and 21 minutes after the test meal. Also notable was that after the first peak, insulin levels in the DIO and the chow groups rose again to a second peak; this was not the case in the DR rats. Because similar patterns of insulin secretion were observed in the chow control and the DIO group, regardless of their diet, this suggests that insulin levels may be more influenced by the actual body weight than by the diet.

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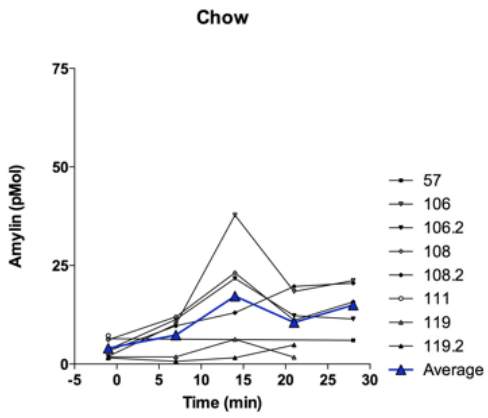

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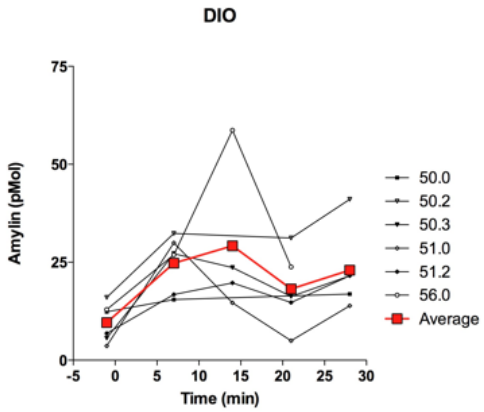

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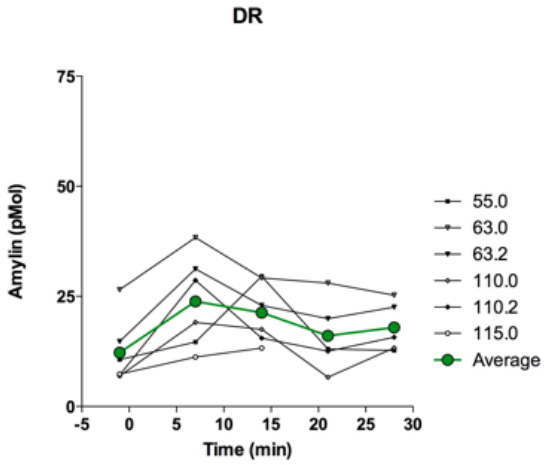

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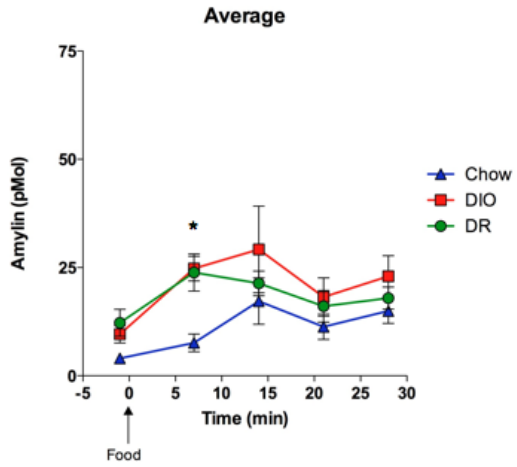

Figure 4.15: Hepatic portal vein amylin concentration in chow control (n=5) (A), DIO (n=3) (B) and DR (n=4) (C) rats after a test meal given at time point 0:00:00. Figure D shows the average of the three groups. Symbols denote significant differences beween the diet regimes; *p<0.05

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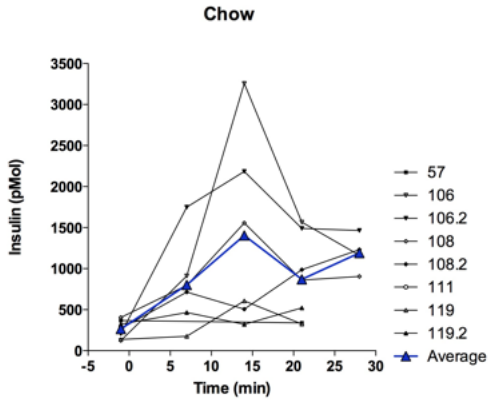

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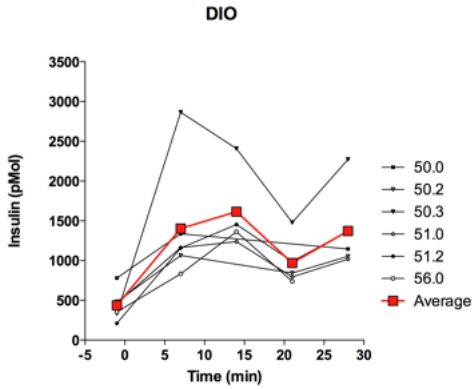

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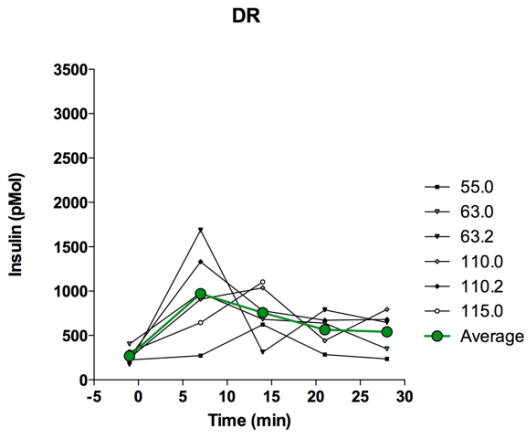

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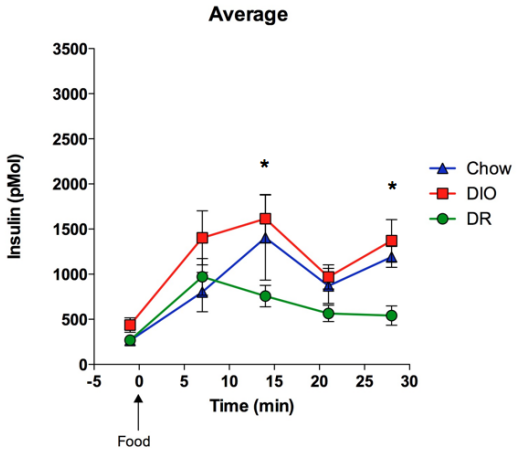

Figure 4.16: Hepatic portal vein insulin concentration in chow control (n=5) (A), DIO (n=3) (B) and DR (n=4) (C) rats after a test meal given at time point 0:00:00. Figure D shows the average of the three groups. Symbols denote significant differences beween the diet regimes; *p<0.05

Figure 4.17 shows the amylin:insulin ratio at the specified timepoints in animals on either standard chow or the high fat diet for about eight weeks. Again, in all three groups we observed a relatively high variability between the individual animals. At all timepoints, the chow-fed control rats demonstrated an amylin:insulin ratio of approximately 0.01, which corresponds well with the amylin:insulin ratios previously reported (Butler et al., 1990; Alam et al., 1992). When comparing the average ratios across the three groups (4.17D), the animals fed the high fat diet, regardless of their DIO or DR phenotype, showed higher amylin:insulin ratios than the chow-fed controls. Interestingly, the DR animals showed the highest ratio values at each sampling time. Because the ratio did not change over time, it seems that the

ratio is affected by the diet group but not by acute changes in hormone secretion during the test meal.

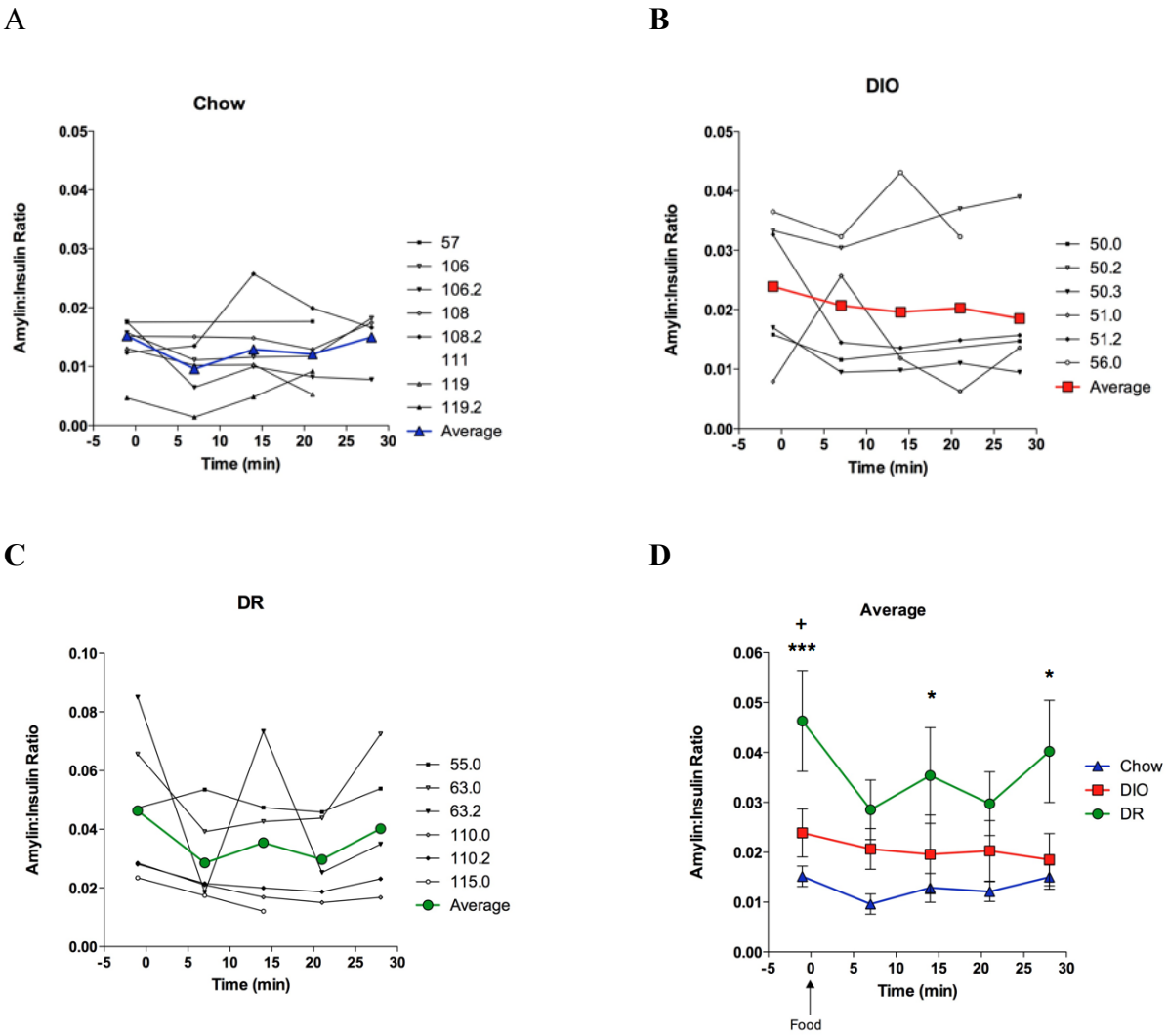

Figure 4.17: Amylin:insulin ratio in chow control (n=5) (A), DIO (n=3) (B) and DR (n=4) (C) rats after a test meal given at time point 0:00:00. Figure D shows the average of the three groups. The different symbols denote significant differences among the three diet groups; * chow vs DR, + chow vs DIO; *p<0.05, **p<0.01

Figure 4.18 shows the average body weights and circulating baseline hormone levels for each group at different time points over the course of the experiment. The results are comparable with those from Experiment 3a. From the beginning of the experiment, DIO animals had a significantly higher body weight than the DR group (retrospect analysis once the three diet groups had been determined) , and also than the chow control group after week 2. In weeks 2

and 5, the chow group also demonstrated significantly higher body weight compared to the DR group (Figure 4.18A).

In all groups we observed an increase in amylin, insulin and leptin levels over time and with increasing body weight. Two-way ANOVA detected a significant effect over time for amylin ($F[df\ 3,154] = 38.59$, $P=<0.0001$), insulin ($F[df\ 3,152] = 53.56$, $P=<0.0001$) and leptin ($F[df\ 3,156] = 48.12$, $P=<0.0001$), as well as a significant effect of the diet group for leptin levels ($F[df\ 1,42] = 7.156$, $P=0.0106$). There was no detectable difference among the three diet groups in amylin levels (Figure 4.18B) at either time point. At the end of the experiment in week 12 and 13, we observed significantly higher insulin levels in the DIO group compared to the DR group (Fig. 4.18C); after five weeks on the high fat diet, leptin levels were significantly higher in the DIO group than in the chow control group (Figure 4.18C).

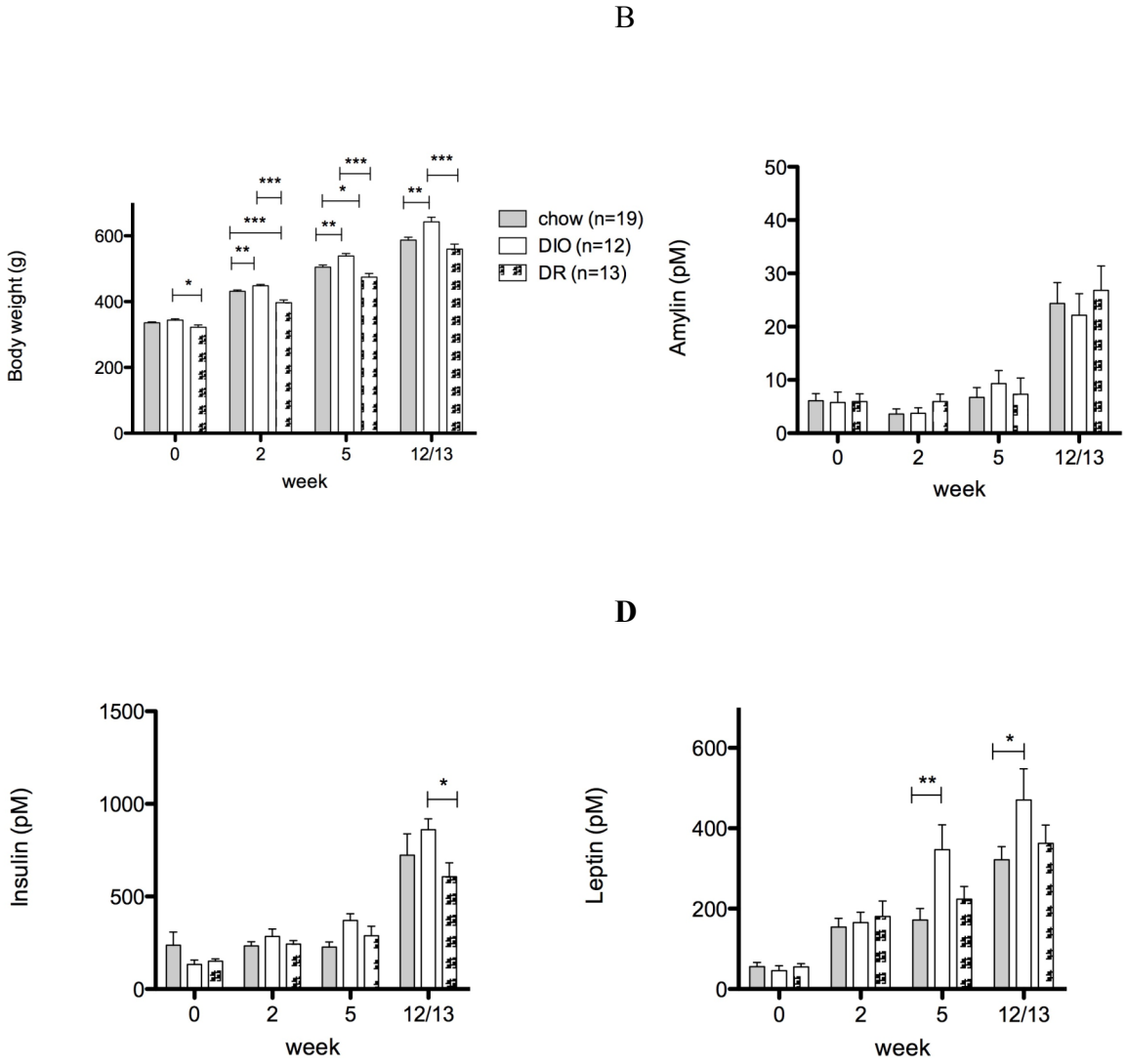

Figure 4.18: Average body weight of each group of rats at each time point after exposure to the high fat diet (A) and mean (± SEM) baseline amylin (B), insulin (C) and leptin (D) levels in rats maintained on chow or the high fat diet for 0, 2, 5 and 13 weeks. Symbols denote significant differences beween the diet regimes; *p<0.05, **p<0.01, ***p<0.001

4.3.3.1 Feeding trials and c-Fos expression, Experiment 3a

To investigate the acute anorectic effect of peripheral amylin in rats maintained on a high fat diet for an extended period of time, a single feeding trial was performed at the end of the experiment (Week 9). Administration of a low dose of 5µg/kg of amylin failed to produce a significant reduction in food intake at 30, 60 or 120 min post-injection, in either the high fat-fed or in the control group (data not shown). The reason for the lack of effect in the control group was surprising and remains at present unexplained.

Using the same rats, we investigated the effect of an acute amylin injection on neuronal activation as gauged by c-Fos protein expression (Figure 4.19). The area postrema was stained for c-Fos protein to investigate neuronal activation following an acute amylin injection (Figure 4.19). In both diet groups, animals were fed ad libitum, with half of the animals treated with 5µg/kg amylin (n=2-3), and half treated with saline (n=2-3). Probably due to the small number of animals per group, there was no significant difference in c-Fos-positive cells two hours post-injection between any group. We saw no effect of diet, but there was a clear trend for an effect of amylin compared to saline (p=0.0523).

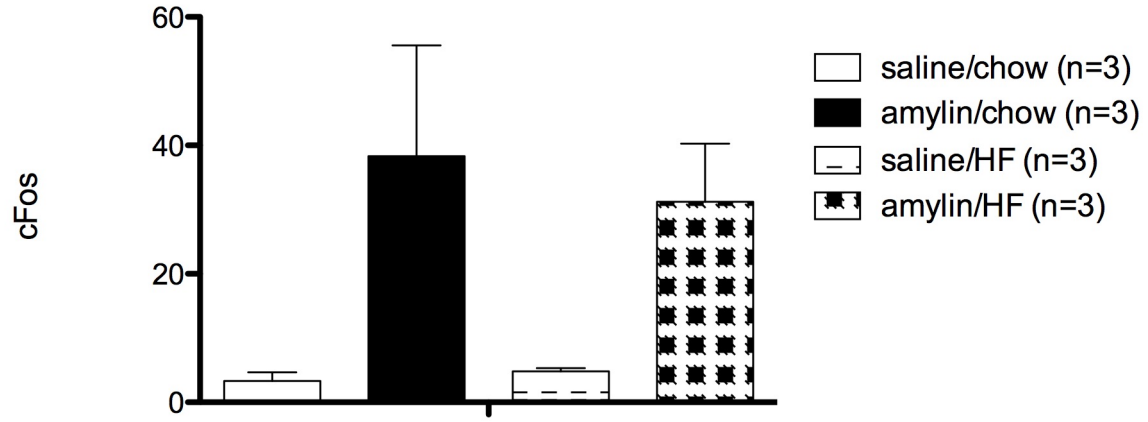

Figure 4.19: Quantification of the number of c-Fos positive cells in the AP of ad libitum fed rats fed either standard chow or a high fat diet, and treated with amylin (5µg/kg) or saline. The animals had been sacrified 2 hours after injection.

4.3.3.1 Feeding trials and c-Fos expression, Experiment 3b

To extend the results of the feeding trial performed in Experiment 3a, three additional acute feeding trials were conducted in a crossover manner. In the first trial performed in week 8 on the respective experimental diets, an amylin dose of 5µg/kg significantly reduced energy intake only in the chow group. The results from the second and third trials in week 9 are shown in Figure 4.20. By increasing the amylin dose to 20µg/kg, a significant decrease in energy intake was also observed in the DIO group at all timepoints and in the DR group 60 and 120 minutes post-injection. After the administration of a high dose of 50µg/kg, we observed results similar to the previous feeding trial, though the effect in the DR group at 60 minutes post-injection was lost.

A B

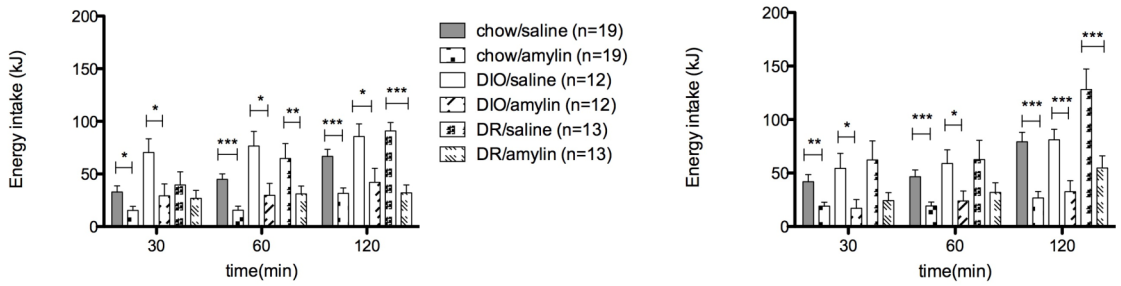

Figure 4.20: Mean (± SEM) cumulative energy intake 30, 60 and 120 minutes after saline or amylin (20µg/kg s.c. (A) , 50µg/kg, s.c. (B)) injection in rats maintained on chow or high fat diet for 7 weeks. Symbols denote significant differences beween saline- and amylin-treated groups within the respective diet regimes; *p<0.05, **p<0.01, ***p<0.001

Similar to experiment 3a, we again tested the effect of amylin on cFos expression in AP neuronal cells in a terminal experiment (Figure 4.21). In all diet groups, animals were fed ad libitum, with half of the animals treated with 5µg/kg amylin (n=3-4) and half treated with saline (n=3-4). The results showed no significant effect of the diet, but there was a significant effect of drug on the number of c-Fos-positive cells in all diet groups (F[df 1, 11] = 16.09, P=0.0020).

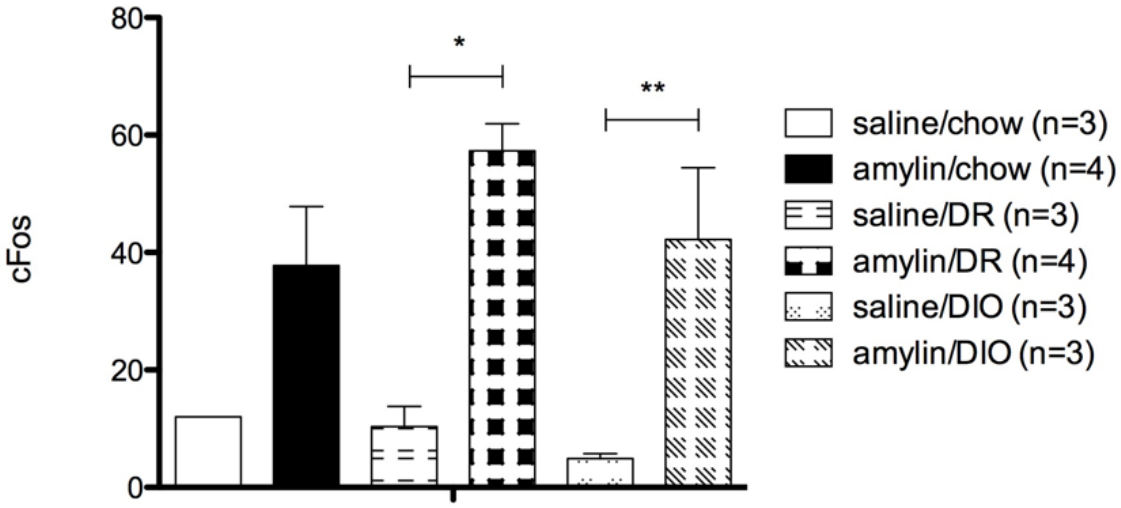

Figure 4.21: Quantification of the number of c-Fos positive cells in the AP of ad libitum fed rats with either standard chow or a high fat diet, and treated with amylin (5µg/kg) or saline. The animals had been sacrified 2 hours after injection. Symbols denote significant differences beween saline- and amylin-treated groups within the respective diet regimes; *p<0.05, **p<0.01

5 DISCUSSION

The aims of this study were to investigate the influence of body composition, of exposure to a high fat diet and of hyperamylinemia, independent of obesity, on amylin sensitivity and secretion. The results of our studies suggest three main points. First, long-term maintenance on a high fat diet, and resulting obesity, appear to attenuate the sensitivity to the acute anorectic effect of amylin. Second, we observed no marked changes in amylin sensitivity in chow fed rats with induced hyperamylinemia that was independent of obesity. Finally, we observed that meal-induced amylin and insulin secretion patterns are differentially affected by obesity and by high-fat diet consumption in DIO, DR and chow-fed control rats.

5.1 The efficacy of amylin to reduce energy intake is decreased after long-term maintenance on high fat diet

To test the hypothesis that diet-induced obesity decreases the rats' sensitivity to the anorectic effects of exogenous amylin, we investigated in the first experiment whether the effect of acute amylin to reduce eating is altered in diet-induced obese or food-restricted rats. To answer this question, we acutely administered several doses of amylin at different time points after the onset of high-fat overfeeding. Additonally, we administered the same doses of amylin to food-restricted animals. Food restriction was expected to lead to moderately lower body weight and body adiposity which may be associated with enhanced sensitivity to amylin's action. To evaluate body composition, we measured the adiposity signals amylin, leptin and insulin, and body fat was assessed directly by computer tomography (CT).

Utilizing high fat diet overfeeding and 80% food restriction relative to the ad libitum chow fed group, we established the overweight and underweight rodent models necessary for this experiment. After 48 days on the high fat diet, we demonstrated that high fat fed rats had a significant increase in body weight compared to chow fed controls. Rats restricted to 80% of the normal food intake had significantly lower body weight than the other two groups during the entire experiment. This difference also remained significant during the refeeding phase, i.e. when the restricted rats received the high fat diet ad libitum for 3 weeks; this followed the 11 week food restriction period. These findings are consistant with previous studies that showed that following prolonged food-restriction, rats maintained a body weight that was

lower than the pre-restriction baseline, even after ad lib refeeding for as long as four months (Brownlow et al., 1993).

To assess body adiposity over the course of the experiment, fasting blood samples of adiposity signals were obtained at two different time points. It is known that leptin is produced and secreted primarily by adipocytes, and that high leptin levels reflect increased adipose mass (Maffei et al., 1995). Furthermore, Pieber *et al.* showed that similar to leptin and insulin, the basal circulating level of amylin also correlates positively with adipose mass across individuals (Pieber et al., 1994). Consistent with these reports, we observed that after 9 weeks on the high fat diet, rats exhibited significant increases in circulating baseline amylin and leptin levels, compared to the chow-fed rats. Unexpectedly, we did not observe an effect of the high fat diet-induced weight gain on fasting insulin levels. These results contrast findings from previous studies that showed that fasting plasma insulin concentrations are positively correlated with body weight in obese and non-obese humans (Bagdade et al., 1967). By the end of the experiment in week 14, the high fat group still showed higher leptin levels than the chow control group. At this timepoint, the previously restricted rats had been fed the high fat diet for three weeks. Interestingly, we found no statistically significant difference between the circualting leptin levels of the previously restricted group and the high fat group, despite the difference in body weight. At this final timepoint, we also did not detect a difference in amylin or insulin levels among groups.

To verify the extent of the diet-induced obesity, body composition was assessed by a CT scan at the end of the experiment. Rats fed the high fat diet had significant increases in visceral fat, subcutaneous fat, and total body fat compared with chow-fed rats. Interestingly, there were no differences in total nor subcutaneous fat mass between the previously-restricted rats that had been switched to high fat diet for only three weeks, and the rats maintained on high fat for 14 weeks; this is consistent with the similar leptin values observed between the two groups at the termination of the study. Only the visceral adipose fat mass of the previously-restricted group remained significantly lower compared to the high fat ad libitum fed rats. It seems that during the three weeks of high fat feeding, the previously restricted rats gained relatively more subcutaneous than visceral fat mass and therefore nearly caught up with the HF ad libitum group in total body fat mass within only three weeks of refeeding; in other words they nearly reached the same level of total body fat mass as observed in the rats fed high fat diet for 14 weeks.

Similar to our results, Brownlow *et al.* (1993) observed a rapid increase of body weight gain during ad libitum refeeding in the rats that had been food restricted to 50 or 75% for two months. After 20 days of refeeding, when the rats had reached approximately 90% of their control weight, body weight gain leveled off and remained significantly lower than the body weights from the control rats during the remainder of the experiment (Brownlow et al., 1993). Interestingly, they observed higher body weight in one group of rats that was food restricted to 50% of control animals. Unfortunately, the distribution of fat mass was not assessed in that report; hence, it is unknown whether the previously-restricted rats also gained relatively more subcutaneous fat. Overall, these and our results suggest that rats placed under food restriction for an extended period, develop very efficient fat-depositing mechanism which leads to rapid weight gain once food is provided ad libitum (Brownlow et al., 1993).

Furthermore, we tested whether the access to the high fat diet or food restriction alter the inhibition of food intake after acute peripheral amylin administration. In the first 10 weeks of the experiment, we observed significant decreases in food intake following amylin injection in rats on the chow control diet and in rats receiving the high fat diet ad libitum. Interestingly, after 11 weeks on the high fat diet, the high fat rats showed a decreased response to a low dose of amylin, whereas the chow control group still ate significantly less after amylin treatment. This result suggests that long-term maintenance on the high fat diet may attenuate the anorectic effect of acute amylin. The change in sensitivity could either be the result of the diet itself, or of the increase in body weight or body fat mass that resulted from the high fat intake. Because we did not see a decrease in amylin sensitivity after high fat feeding for up to 8 weeks, it seems unlikely that the change in amylin sensitivity is due solely to the fat content of the diet. It seems more likely that this change in sensitivity is based on the resulting change in body weight or body composition. This notion is supported by the fact that at the point in time when the anorectic effect of amylin was lost in the high fat group, the difference in body weight between this group and the chow control became significant.

Covasa and colleagues observed a similar effect of exogenous amylin in rats fed a HF diet for three weeks; there was no difference in the sensitivity to the anorectic effect of amylin between animals adapted to either a low or high fat diet (Covasa and Ritter, 1998). Interestingly, using the same paradigm, Covasa and colleagues did observe a significant attenuation of the CCK effect in Sprague-Dawley rats after maintenance on high fat diet. While the highest dose of CCK tested (1µg/kg) produced a reduction of food intake that did

not differ between low- and high-fat fed rats, lower doses of CCK, while still effective in high fat-fed rats, were significantly less powerful at reducing food intake, compared to low fat-fed rats. It is unfortunate, however, that Covasa did not carry out the study for a longer time period to assess if sensitivity to CCK (and amylin) changes with prolonged maintenance on high fat diet. Additionally, rats were fed a high fat diet for 2 weeks containing 30% kJ from fat, and unlike in our study, rats were food deprived for 17 hours prior to treatment. Thus, while these data are interesting, and consistent with our study, in that short-term exposure of rats to a high fat diet does not reduce sensitivity to the acute anorectic effect of amylin, differences exist between the study designs that make it difficult to compare them directly.

In contrast to the DIO rats, food-restricted rats only responded to an acute amylin injection when the animals were pre-fed for one hour with 50% of their daily amount of food and when they received a comparably high dose of amylin (50µg/kg). Because the anorectic effect was only observed after pre-feeding, we believe that the drive to eat after prolonged food restriction may simply have been too strong, and likely masked the anorectic properties of amylin. It seems that decreased body weight, at least under these test conditions of acute amylin injections does not appear to increase sensitivity to amylin. These findings may seem to contrast previous studies that showed that food restriction for up to 8 months of age increases whole body insulin sensitivity (Escriva et al., 2007). However, it is well-established that food deprivation produces a number of neural and physiological responses aimed at stimulating eating and correcting for the impinging energy deficit; this most likely contributed to our findings. Plasma insulin is one of the signals that is reduced in food-deprived rats to allow the mobilization of stored energy from adipose tissue and to promote gluconeogenesis (Seeley et al., 1996). Additionally, the concentration of the hunger signal ghrelin is known to increase after fasting and it is also known that a loss in body weight leads to an increase in circulating ghrelin concentration (Hansen et al., 2002; Cui et al., 2008). The upregulation of these and other mechanisms in food-deprived rats may explain why we did not observe an anorectic effect of acute amylin in the food restricted animals, at least with doses that reduce eating in non-restricted animals.

Another explanation for changes in amylin sensitivity between obese and lean rats could be the interaction between amylin and leptin. It is known that a co-infusion of amylin and leptin reduces body weight and adiposity synergistically in DIO prone rats (Trevaskis et al., 2008). Additionally, it has been shown that peripheral administration of amylin restores leptin

sensitivity in rats and humans (Roth et al., 2008). These findings suggest that an interaction exists between exogenous amylin and the prevailing circulating leptin values. It is possible that exogenous amylin only shows its anorectic effect in a certain range of circulating leptin levels. Although speculative, this may explain why we observed a reduced sensitivity to the anorectic activities of acute amylin in both obese rats, with high circulating leptin levels, and in restricted rats, with low circulating leptin levels.

5.2 Hyperamylinemia alone does not cause amylin insensitivity

In the first series of experiments, we had observed a decrease in amylin sensitivity after long-term exposure of rats to a high fat diet, which resulted in increased body weight, fat mass, hyperleptinemia and hyperamylinemia. Because it remained unclear which of these factors primarily contributed to decreased amylin sensitivity after prolonged high fat consumption, we investigated in the next experiment whether hyperamylinemia alone, independent of other obesity-related factors, causes a decrease in the sensitivity to acute amylin administration. In addition to becoming obese, hyperinsulinemic and hyperleptinemic DIO rats fed a high energy diet also show a decrease in insulin and leptin sensitivity (Levin et al., 1997; Levin and Dunn-Meynell, 2002). Because obesity is also associated with hyperamylinemia, we hypothesized that elevated levels of circulating amylin dose might contribute to decreased amylin sensitivity.

To address this hypothesis, circulating baseline amylin levels were clamped to achieve the physiological levels found in lean and obese rats; rat amylin was chronically infused using osmotic minipumps. The administered amylin doses were chosen based on circulating levels observed in our previous studies and also based on previously published work (Mack et al., 2007; Trevaskis et al., 2008). Thus, by chronically infusing 5 or 10µg/kg/day for seven days, we achieved average baseline circulating amylin levels of 31.2 ± 4.1 and 37.2 ± 4.7 pM, respectively which were significantly higher than the baseline concentration in lean rats (16.5 ± 2.7 pM); these values also correspond well with those observed in our third experiment (e.g. between 15 and 40 pM). During each of these clamping periods, the rats received three acute injections of various doses of amylin or saline to assess the acute anorectic effect of amylin under conditions of differing underlying baseline amylin levels. To limit the influence of external factors, rats were maintained on standard chow, and body weight at the start of each

infusion period was similar in all groups, even though body weight was slightly reduced during amylin infusions.

In contrast to our hypothesis, we observed no marked difference in amylin sensitivity among the groups with different background circulating amylin levels. Thus, regardless of the level of circulating amylin, acute peripheral amylin administration decreased food intake in a dose-dependent manner, and this effect was similar under all conditions. These results suggest that hyperamylinemia alone, without the impact of obesity or other obesity associated parameters, does not cause a marked decrease in amylin sensitivity. It is important to note, however, that two hours after amylin treatment with a dose of 5µg/kg, the anorectic effect of amylin was no longer detected in the animals with the moderate (5µg/kg/24hr) and high (10µg/kg/24hr) circulating baseline amylin levels, though the effect was present in the control rats receiving baseline saline infusion. This might suggest the presence of a subtle reduction in amylin sensitivity in rats receiving chronic amylin infusion, and that after two hours the concentration of the acute administered amylin was too low to induce a further decrease in food intake in animals with elevated baseline circulating amylin values. Therefore, it is possible that by administering a lower dose of acute exogenous amylin, a decrease in amylin sensitivity might have been uncovered under such conditions.

In earlier studies, chronic subcutaneous or third ventricular infusion of amylin produced a transient, dose-dependent decrease in daily food intake and a decrease in body weight gain (Rushing et al., 2000; Olsson et al., 2007). Mack *et al.* showed that amylin (10µg/kg/day) infused subcutaneously for four weeks decreased food intake and body weight in rats fed a high fat diet for five weeks (Mack et al., 2007). Consistant with these findings, our rats receiving exogenous amylin showed a tendency to gain less body weight, and the effect seemed to be dose-dependent. We also detected a tendency for lower baseline/24 hour energy intake in animals with high background circulating amylin levels. However, probably because of the low doses of administered amylin, the difference across the groups for the effect on body weight gain or baseline food intake did not reach significance. In a similar study by Arnelo *et al.,* the minimal effective dose for suppressing daily food intake was ~ 32.3µg/kg/day (7pmol/kg/min) which produced plasma levels of amylin of approximately 80 pmol/l (Arnelo et al., 1996), i.e. much higher than the physiological levels observed in DIO rats and much higher than the concentrations in our experiment. Even though our elevated levels of amylin, which were still in the physiological range, did not produce dramatic

changes in body weight and food intake, our results do provide further evidence that amylin may play a role in the long-term regulation of energy balance, similar to well known adiposity signals, like insulin and leptin.

To interpret the result of our experiments, another point to consider is that chronic hyperamylinemia may affect circulating levels of insulin or leptin. It has been shown that insulin secretion was increased by the administration of an amylin antagonist in isolated rat islets (Wang et al., 1999), suggesting that an ineraction exists between amylin and insulin secretion. This idea was further supported by findings of Salas *et al.* that insulin secretion in the rat pancreas was suppressed by high doses of amylin (Salas et al., 1995). Additionally, Gebre-Medhin *et al.* observed that amylin deficient mice (IAPP-/-) showed increased plasma insulin responses following glucose administration, whereas baseline insulin and glucose levels were normal (Gebre-Medhin et al., 1998). Together, these data demonstrate that high amylin levels may feedback to suppress insulin secretion. While we did not measure insulin values in our experiment, it would be interesting to investigate if a sustained increase in circulating amylin values influenced circulating insulin levels and which role this may play in the eating inhibiting effect of amylin. Furthermore, because of the known interaction between amylin and leptin (Roth et al., 2008; Trevaskis et al., 2008), it would be interesting to determine whether an increase of amylin, independent of obesity, alters circulating leptin levels. While a recent study has shown that leptin levels do not regulate circulating amylin levels (Hwang et al., 2008), the reverse effect has not been investigated.

5.3 Meal-induced amylin secretion in lean and obese rats

In the last series of experiments, we investigated the meal-induced amylin secretion pattern in obese and lean rats. Previous studies investigating amylin and insulin secretion had typically been perfomed in isolated rat pancreas or in vivo following the intravenous stimulation with glucose or arginine. For example, studies from Ogawa *et al.* investigated amylin secretion from the perfused rat pancreas under conditions known to stimulate the secretion of insulin (Ogawa et al., 1990); they observed a co-secretion of insulin and amylin with similar relative potencies after glucose or arginine stimulation. Studies in humans have shown that during intravenous glucose infusion, amylin is secreted in regular cyclic oscillations (Juhl et al., 2000), and that morbidly obese humans exhibit a wide range of amylin-to-insulin secretory ratio values (Blackard et al., 1994). However, none of these studies determined how amylin

and insulin secretion change during and immediately following a physiological meal. Our study demonstrates for the first time the patterns of meal-induced amylin and insulin secretion in obese and non-obese rats.

First, we successfully established the DIO model in our laboratory. In accordance with findings from Levin and colleagues in outbred male Sprague-Dawley rats receiving a moderately high fat diet for two weeks (Levin et al., 1983, 1986; Levin and Keesey, 1998), we also observed a clear separation between weight-gaining diet-induced obese (DIO) rats and their diet resistant (DR) counterparts. Upon designation of the phenotypes, retrospective analysis of the average baseline body weights showed that DIO animals had been significantly heavier than DR animals even before access to high fat diet. This phenomenon, which has been previously observed by others (Levin et al., 1987) suggests that the bimodal pattern of body weight gain is already present during standard chow feeding. After two weeks on the high fat diet, all groups were significantly different from each other, as the phenotypes were fully unmasked by the access to the high fat diet.

Consistent with the findings in our first experiment, we observed an increase in amylin, leptin and insulin concentrations that paralleled the increase in body weight over the course of the experiment. While we did not detect a clear difference in baseline amylin levels among the groups, leptin levels in DIO rats increased significantly compared to chow control animals after five weeks on the high fat diet; this is similar to what had been previously reported in outbred rats as early as four weeks on a high fat diet (Levin and Dunn-Meynell, 2002). In respect to insulin levels, Levin observed that within two weeks on the high fat diet, DIO-prone rats developped glucose intolerance and hyperinsulinemia after an overnight fast (Levin et al., 1997). In contrast to these finding, our DIO rats showed higher fasting insulin levels only after 12 weeks on the high fat diet when compared to the DR animals; further we never detected higher insulin levels in DIO compared to chow control animals. One key difference that may have contributed to the discrepant results was that the Levin study utlized Sprague-Dawley rats that were selectively bred for DIO and DR phenotypes, rather than the outbred rats that we used. Selectively bred DIO rats begin showing signs of obesity even on standard chow, and these symptoms are exacerbated when high energy diet is made available (Levin et al., 1997).

It is thought that decreased insulin sensitivity subsequent to the weight gain results in a compensatory increase in insulin secretion in the basal state and in response to meals in order to maintain normal glucose homeostasis (Clegg et al., 2005). At the time when we looked at meal-induced amylin and insulin secretion pattern, we observed no differences in circulating amylin and insulin baseline levels among the three groups. However, when looking at the pattern of portal vein hormone concentration of amylin and insulin in twelve-hour food deprived rats, there was a tendency for elevated baseline amylin levels in the two groups fed the high fat diet (DIO and DR), compared to the chow fed control animals. Fasting portal vein insulin levels, on the other hand, were indistinguishable among the groups.

Shortly after meal intiation, we observed a biphasic increase in amylin levels with a first peak within 15 minutes after meal onset, and a second increase starting 20 to 25 minutes later. This pattern of secretion was observed in all three groups. Seven minutes after meal onset the meal, the high fat-fed animals had significantly higher amylin levels than the control animals. Thus, the meal induced amylin secretion seems to be more influenced by the fat content of the diet than by the current body weight or composition because body weight in chow fed controls and DR rats was similar. For insulin secretion, DIO and the chow fed animals showed a similar secretion pattern as found for amylin. The first peak in insulin levels appeared also within the first 15 minutes after meal onset, and the second peak started 20 to 25 minutes later. In the DR animals, the initial peak was observed 7 minutes after meal onset, at which point the insulin levels were not different from the other two groups. Interestingly, after this first small peak, insulin levels continuously decreased without rising to a second peak. This resulted in significantly lower insulin levels at the 14- and 28-minute timepoints compared to the DIO and the control rats. Hence, unlike amylin, insulin levels seem to be less influenced by the nature of the diet but that the actual body weight of the animals possibly their adiposity may be responsible for the differences in the secretion pattern of insulin.

To investigate the influence of the diet composition on meal-induced amylin secretion, we believe that further experiments need to be performed. One possibility would be to switch DIO rats back to chow for some weeks before the experimental blood sampling after a test meal. By doing so, the animals could still be identified as DIO, but would receive standard chow diet at the time of testing, and the factor of the fat content of the diet at the time of testing would be eliminated.

Two other issues emerged during analysis of the data. First, we observed a large inter – individual variation in amylin and insulin secretion patterns in all three groups. It can be speculated that this variability may be attributed to the pulsatile manner in which amylin and insulin are secreted. Matveyenko *et al*. observed that, consistent with canine and human studies, insulin in rats is secreted in a high-frequency pulsatile manner, with pulse intervals of approximately 4-5min (Matveyenko et al., 2008). Based on these data, it was postulated that in the fasting state, the liver is exposed to insulin oscillations of ~400-600 pmol/l (Matveyenko et al., 2008). Though amylin pulsatility has not been confirmed in rats, humans exposed to constant glucose administration demonstrate a regular cyclic oscillation of circulating amylin concentration (Juhl et al., 2000). Furthermore, because amylin and insulin are contained in the same secretory vesicles (Hartter et al., 1991; Bai et al., 2006), it is likely that amylin, like insulin, is also secreted in a pulsatile manner in rats. Thus, the exact time point in the secretory oscillation cycle at which each blood sample was collected could markedly influence measured amylin and insulin values, thus resulting in high variability. The second technical matter was that it was not always possible to obtain a blood sample from each animal at all timepoints. Such missing values present problems when performing analyses such as area under the curve (AUC).

Despite the variabilty among rats, and because we were able to measure amylin and insulin in the same blood sample, we also assessed the amylin-to-insulin secretion ratio. It is known that high concentrations of glucose can lead - by unknown mechanism - to an increase in the amylin:insulin molar ratios in the perfused rat pancreas (Inoue et al., 1992); further, morbidly obese humans exhibit amylin:insulin secretory molar ratios anywhere between 0.02 to 0.16 (Blackard et al., 1994). Our results show that the animals on the high fat diet had a higher amylin:insulin ratio compared to the chow fed control animals. While the ratio observed in chow-fed rats was maintained around 0.015 throughout the sampling period, the ratio in DIO rats was slightly higher at around 0.025; in DR rats, the ratio actually ranged from 0.03 to 0.045. Hence it seems that the DIO and DR test meal, which was a high fat pellet with a sucrose component, may have contributed to a relatively higher amylin secretion compared to insulin. An earlier study showed that high doses of amylin actually inhibit insulin secretion from rat pancreas (Salas et al., 1995). Based on these results, it seems possible that in high fat-fed rats that secrete more amylin, insulin secretion may be slightly suppressed, resulting in elevated amylin:insulin secretion ratios, especially in the DR group. However, this idea needs to be tested in a proper experiment.

Finally, we assessed if the DIO and DR phenotypes produce changes in the feeding responses or the central neuronal activation pattern to an acute injection of amylin. Similar to the findings from the first experiment, i.e. when rats ingested a high fat diet with 60% fat, animals fed the diet with a fat content of about 30% for ten weeks showed a decrease in amylin sensitivity after a low dose of amylin (5µg/kg). With a moderate (20µg/kg) and a high (50µg/kg) dose of amylin, only the DR group seemed to be less sensitive to the acute anorectic action of amylin. Of note, we did not detect a difference in the number of c-Fos activated cells in the AP following low dose amylin (5µg/kg) treatment across the three groups. This suggests that if there were altered sensitivity in DIO and DR rats, this was not detectable based on cFos immunocytochemistry, i.e. we did not detect a change in neuronal activation in the AP using this technique.

The sensitivity to other peptides that induce satiation has also been investigated in rats fed high fat diet, or in rats classified as either DIO or DR. Rats maintained on a high fat diet for as short as two weeks showed decreased sensitivity to the eating inhibitory effects of CCK. Interestingly it has also been described that DIO-prone rats fed a standard chow diet show increased sensitivity to the anorectic effect of CCK treatment (4µg/kg, i.p.), as well as an increase in the number of c-Fos positive cells in the NTS, compared to rats that are DR-prone (Swartz et al., 2010). However, DIO-prone, chow-fed rats show significantly reduced sensitivity to the anorectic response of centrally administered leptin (Levin and Dunn-Meynell, 2002). Thus, both maintenance on a high fat diet and the presence of a DIO or DR phenotype can differentially affect the sensitivity to various peptides involved in the control of food intake.

5.4 Concluding Summary

In summary, our results show that long-term high fat feeding influences amylin sensitivity and secretion in diet-induced obese rats. First, we observed that rats maintained on a high fat diet for more than 11 weeks were less sensitive to the anorectic action of acute exogenous amylin. Interestingly, the rats showed a similar response effect to amylin in the first weeks on the high fat diet, as control rats. This leads to the assumption that it may not be the composition of the diet itself which decreased amylin sensitivity under these conditions, but rather the actual body weight or adiposity of the animals which was achieved after prolonged high fat feeding. Furthermore in the second study we saw a dose-dependent decrease in food

intake after acute amylin application, independent of the underlying baseline amylin concentrations that was elevated to that of obese rats by chronic amylin infusion (5 or 10 µg/kg/day). Hence the reduced sensitivity in obese animals is most likely not a direct result of hyperamylinemia. We also obtained promising early findings that suggest that decreased amylin sensitivity may not be occuring at the primary central target, the area postrema, but rather downstream in the neuronal pathway. Finally, the differences in amylin and insulin secretion pattern between DIO, DR and chow-fed rats indicate that not only baseline hormone levels are influenced by body composition; the meal induced amylin secretion also seemed to be influenced by the composition of the diet, but less by the actual body weight or body composition.

The fact that long-term maintenance on a high fat diet reduced amylin sensitivity and that amylin secretion may be influenced by the fat content of the diet, is an important finding for the future of amylin as a therapeutic agent. Amylin-based therapy, in particular when combined with leptin treatment, seems to be a promising therapy for overweight humans. Because obesity and the associated development of type 2 diabetes is mostly the result of overeating combined with other environmental and genetic factors, our data are especially pertinent. Thus, if diet itself influences the sensitivity to amylin, then combination treatments with amylin and leptin could potentially be improved by an additional modification in the amount of fat consumed in one's diet. While additional research is clearly needed to explore such a hypothesis, our data provide considerable evidence of obesity-related factors that modify amylin sensitivity and secretion.

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